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## Evaluation of high hydrostatic pressure, meat species, and ingredients to control *Listeria monocytogenes* in ready-to-eat meats

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**Evaluation of high hydrostatic pressure, meat species, and ingredients to  
control *Listeria monocytogenes* in ready-to-eat meats**

by

**Kevin Lee Myers**

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

Co-majors: Meat Science; Food Science and Technology

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2012

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## GENERAL INTRODUCTION

### Introduction

*Listeria monocytogenes* is a foodborne pathogen that is unique in the fact that it can grow at refrigerated temperatures and potentially cause illnesses and death in those that unknowingly consume it. *L. monocytogenes* is also an organism that has a high virulence and therefore a relatively high level of deaths occur in patients inflicted by the disease listeriosis, which is caused by *L. monocytogenes*. Due to these facts the Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) have deemed *L. monocytogenes* an adulterant and have a zero-tolerance policy for the pathogen in ready-to-eat food items. Control of *L. monocytogenes* in meat items has been centered on use of ingredients and processes to decrease potential contamination of *L. monocytogenes* and to limit its growth during refrigerated storage. A great deal of research has been conducted on *L. monocytogenes* control, but recalls of products contaminated with *L. monocytogenes* continue and further research is still needed to improve control measures and minimize food safety risks for consumers.

Research on the effect of meat species on high hydrostatic pressure (HHP) processing of ready-to-eat meat products has not been conducted. Also, natural foods continue to gain consumer appeal, but the restricted ingredient requirements for these products may allow for increased food safety risks in these items and safety of these products with and without the use of HHP are areas that further research is needed. Lastly, the effect of the combination of commercially-available antimicrobial ingredients and HHP is an area that could also use further research to maximize food safety while

reducing the overall cost to consumers.

## **Dissertation Organization**

This dissertation starts with a short introduction of the overall research and then is broken down into five separate chapters. The first, second, and third chapters are organized in the style of the journal *Meat Science*. The fourth chapter is organized in the style of the *Journal of Food Protection*. The first chapter is an overall review of relevant prior literature on research in the areas of *L. monocytogenes* incidence, *L. monocytogenes* virulence, foods at risk for growth of *L. monocytogenes*, control efforts for *L. monocytogenes*, USDA requirements, ingredient effects on *L. monocytogenes* growth, and processing effects on *L. monocytogenes* growth. The second chapter is a manuscript titled “Growth of *Listeria monocytogenes* in RTE ham and turkey with and without use of high hydrostatic pressure.” The third chapter is a manuscript titled “Effects of sodium nitrite and concentration of pre-converted vegetable juice powder on growth of *Listeria monocytogenes* in RTE sliced ham with and without high hydrostatic pressure.” The fourth chapter is titled “Effects of High Hydrostatic Pressure, Antimicrobial Ingredients, and Antimicrobial Sprays on Growth of *Listeria monocytogenes* in Conventional and Natural Formulations of RTE Sliced Ham.” The fifth and final chapter is an overall summary and conclusions from the research with recommendations for further research that is needed.



## CHAPTER 1 - Literature Review

### Foodborne Illness

The United Nations Food and Agriculture Organization (FAO) estimated that three million people die each year from foodborne or waterborne causes, with millions more developing less severe illnesses (FAO, 2011a). Scallan, Hoekstra, et al. (2011) reported that foodborne pathogens caused 9.4 million illnesses and 1,351 deaths per year over the past decade in the United States. Of the pathogens reported by Scallan, Hoekstra, et al. (2011), the greatest numbers of hospitalizations were caused by nontyphoidal *Salmonella*, Norovirus, and *Campylobacter* with the greatest number of deaths caused by nontyphoidal *Salmonella*, *Toxoplasma gondii*, and *Listeria monocytogenes*. Lynch, Painter, Woodruff, and Braden (2006) reported on results of a total of 6,647 cases of foodborne disease outbreaks from 1998 to 2002, and showed that 54% of deaths were caused by *L. monocytogenes*. Scallan, Griffin, Angulo, Tauxe, & Hoekstra (2011) reported that an additional 38.4 million foodborne illnesses and 1,686 deaths per year in the United States are caused by foodborne illnesses due to unspecified agents. Immunocompromised individuals such as the elderly or pregnant women are at a much greater level of risk for infection by some pathogens and therefore it is probable that a lesser number of ingested organisms may supply an infectious dose to this immunocompromised group compared to those that do not have underlying health conditions (Vazquez-Boland, Kuhn, et al., 2001). At the same time that the world population is continuing to expand, the U.S. population is aging, as 12.4% of Americans were age 65 or older in 2010 (Census.gov, 2011a) and those greater than age 65 in the

U.S. is projected to grow to 20.7% of the population by 2050 (Census.gov, 2011b), thus putting an increased percentage of people into the immune compromised group. FAO (2011b) has estimated that global demand for food will nearly double by the year 2050. Therefore, developing processes to improve food safety, while at the same time minimizing food waste, will be an important component for safely meeting the projected increased demand for food.

## **Listeria Overview**

### **History**

*Listeria monocytogenes* (*L. monocytogenes*) is the only species within the genus *Listeria* that is a pathogen of concern for humans (Kathariou, 2002). *L. monocytogenes* is a pathogen that can infect a wide range of host tissues but often targets the growing fetus in pregnant women and the central nervous system of adults (Vazquez-Boland, Kuhn, et al., 2001). The disease listeriosis is an infection caused only by the pathogen *L. monocytogenes* (Hof & Hefner, 1988; Dussurget, Pizarro-Cerda, & Cossart, 2004). The discovery that *L. monocytogenes* could be transmitted from food to humans was conclusively made in the 1980's as epidemiologic and laboratory investigations were able to link outbreaks of listeriosis to specific food items (Schlech et al., 1983; Fleming et al., 1985; Linnan et al., 1988). *L. monocytogenes* can grow at refrigerated temperatures and thus poses a risk for refrigerated ready-to-eat foods (Farber & Peterkin, 1991). *L. monocytogenes* contamination of foods has been linked to both sporadic cases and large-scale outbreaks of listeriosis (Kathariou, 2002). Commercial cooking processes for meats are designed to provide an adequate kill of *L. monocytogenes* during lethality cooking of

finished products, but contamination with *L. monocytogenes* can occur after cooking as the product is portioned and packaged in its final container (Sauders & Wiedmann, 2007).

Several factors are involved in the ability for invasive foodborne listeriosis to appear in hosts. These include the total numbers of *L. monocytogenes* organisms consumed, the virulence of the specific strain (or strains), and the overall health of the consumer (Schuchat, Swaminathan, & Broome, 1991). *L. monocytogenes* has the ability to enter, survive, and multiply as phagocytic or non-phagocytic cells (Cossart & Lecuit, 1998). This ability to survive and attack in human hosts can lead to the development of listeriosis (Cossart & Lecuit, 1998). There are three barriers in human host that are normally impenetrable by bacterial pathogens – the intestinal barrier, the blood-brain barrier, and the placental barrier – but *L. monocytogenes* also has the capability to cross all three of these barriers in human hosts and cause listeriosis (Lecuit & Cossart, 2002). This ability of attacking human hosts is believed to be one of the main reasons that *L. monocytogenes* has a greater mortality rate than other pathogenic bacteria (Cossart & Lecuit, 1998). The two primary groups that are at greatest risk for listeriosis are the elderly (>60 years of age) and neonates (Kuhn, Scortti, & Vazquez-Boland, 2008). The mortality rate for listeriosis infection was reported at 26-50% in various papers which evaluated listeriosis cases from 1967 to 1985 (Fleming et al., 1985; Linnan et al., 1988; McLauchlin, 1990b; McLauchlin, 1990c; Schuchat et al., 1991). The updated foodborne pathogen data reported by Scallan, Hoekstra, et al. (2011) listed the human death rate resulting from *L. monocytogenes* at 15.9%.

Gray and Killinger (1966) reported that the organism now known as *L.*

*monocytogenes* may have been first encountered by Hülphers, in Sweden in 1911.

Murray, Webb, & Swann (1926) witnessed the sudden death of six rabbits, with the Gram-positive bacteria isolated from the rabbits having a large mononuclear leukocytes and Murray named the organism *Bacterium monocytogenes* (Gray & Killinger, 1966). Upon discovery of the same organism in 1927, J.H.H. Pirie chose the name *Listerella hepatolytica* (Gray & Killinger, 1966). Pirie (1940) then chose the accepted name of *Listeria* for the genus, and *monocytogenes*, as suggested by Murray et al. (1926), remained the species name (Gray & Killinger, 1966).

After Murray et al. (1926) discovered the organism, now known as *L. monocytogenes*, in rabbits, Gill (1933) identified the organism in sheep, and for several decades listeriosis was considered an animal disease (Schuchat et al., 1991). Gray and Killinger (1966) noted that since the bacterium now known as *L. monocytogenes* was discovered by Murray et al. (1926), very little additional information was reported about the organism over the next 40 years. However Murray et al. (1926) had the foresight to describe the organism as a “potential menace” and an “indiscriminate killer.”

*L. monocytogenes* has been isolated from many different sources including water (El-Taweel & Shaban 2001; Watkins & Sleath 1981; Sauders et al. 2006), soil (Weis & Seeliger 1975; Sauders et al. 2006), silage (Caro et al.; 1990; Fenlon, 1986; Ryser, Arimi, & Donnelly, 1997), vegetation (Sauders et al., 2006; Welshimer & Donker-Voet, 1971), sewage (al-Ghazali & al-Azawi, 1988; Colburn, Kaysner, Abeyta, & Wekell, 1990), human stool (Sauders, et al. 2005), and animal feed (Skovgaard & Morgen, 1988; Wiedmann, Arvik et al., 1997).

Human cases of listeriosis were initially linked to contact with animals, which

were considered a primary reservoir for *L. monocytogenes* (Owen, Meis, Jackson, & Stoenner, 1960; Schuchat et al., 1991). Gray and Killinger (1966) pointed out inconsistencies with this theory, noting a great number of listeriosis cases occurred in urban areas where those infected had very limited contact with animals. It was not until many years later that large outbreaks of human listeriosis were positively linked to food sources (Schlech et al., 1983; Fleming et al., 1985; Ho, Shands, Friedland, Eckind, & Fraser, 1986; Linnan et al., 1988).

Farber and Peterkin (1991) stated that 5 to 10% of the human population could be carriers of *L. monocytogenes* without showing any signs of infection. Kathariou (2002) also stated that some humans are probably non-symptomatic carriers of *L. monocytogenes*. *L. monocytogenes* is ubiquitous in nature and has the unique ability among pathogens of growing, although at a slower rate, at refrigerated temperatures, making it a pathogen of highest concern for safety of ready-to-eat foods that are consumed without re cooking (Farber & Peterkin, 1991; Kathariou, 2002).

### **Cellular structure of *Listeria***

The outer cell envelope of *Listeria* and other Gram-positive bacteria consists of a cytoplasmic membrane surrounded by a rigid cell wall (Shockman & Barrett, 1983). The cell wall contains peptidoglycan and secondary cell wall polymers consisting of mostly teichoic and teichuronic acids (Archibald, 1985; Navarre & Schneewind, 1999; Schaffer & Messner, 2005). The cell wall of *Listeria* strains is composed of about 30-35% peptidoglycan and 60-70% teichoic acid (Wagner & McLauchlin, 2008). Teichoic acids are covalently attached to the peptidoglycan layer and teichuronic acids are more loosely linked to the cell wall via a lipid anchor moiety (Fiedler, 1988; Desvaux & Hebraud,

2008). The peptidoglycan plays a key role in protecting the cell from changes in osmotic pressure (Fiedler, 1988).

### **Infectious dose, risk to immunocompromised, and virulence**

#### *Listeria* species and serotypes of *L. monocytogenes*

The *Listeria* genus can be divided into six species – *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, and *Listeria grayi* (Rocourt & Buchrieser, 2007). Animal listeriosis may be caused by the animal pathogen *Listeria ivanovii*, but this organism does not normally cause human infections (Wiedmann, Arvik, et al., 1997), whereas *Listeria monocytogenes* has been shown to be the only *Listeria* species of concern for potential human infections (Kathariou, 2002). Investigations of clusters of illnesses in the early 1980's lead to the conclusion that *L. monocytogenes* could be transmitted by contaminated food (Schlech et al., 1983) and is capable of causing outbreaks of listeriosis (Kathariou, 2002). Casadevall and Pirofski (1999) classified *L. monocytogenes* as a “Class 2” pathogenic organism, capable of causing infection and illness in normal hosts, but more often associated with causing disease in those with compromised immune systems. Painter and Slutsker (2007) reported that listeriosis occurs at a greater rate in the elderly, newborns, and pregnant women as well as others that have underlying conditions causing them to be immunocompromised.

*L. monocytogenes* utilizes the nutrients within food products for survival/growth, and then human consumption of these food items containing *L. monocytogenes* can cause listeriosis in some consumers (Lianou & Sofos, 2007; Kathariou, 2002). Unlike other pathogens such as norovirus or *Staphylococcus aureus*, no evidence has directly implicated *Listeria* in any food-worker related outbreaks (Todd, Greig, Bartleson, & Michaels,

2008), however, Endrikat et al. (2010) suggested that increased retail handling of ready-to-eat meats may be a factor in why there is a greater prevalence of *L. monocytogenes* in retail-sliced meats than in those pre-packaged by the manufacturer.

Suyemoto, Spears, Hamrick, Barnes, Havell, and Orndorff (2010) found that in mouse models, susceptibility of the placenta to *L. monocytogenes* infection occurred very early in gestation and the authors theorized that the embryo could provide a more protective environment for *L. monocytogenes* replication compared with the maternal environment. They also reasoned that a more fully developed placental barrier later in pregnancy may inhibit *L. monocytogenes* uptake by the embryo. Bakardjiev, Stacy, and Portnoy (2005) showed in a guinea pig model that there was a greater than 1000-fold increase in the number of *L. monocytogenes* in the placenta within 24 hours after inoculation. The authors theorized that changes in maternal immunity that allow tolerance of the fetus may also play a role in providing a protective environment for *L. monocytogenes* growth as well.

Glaser et al. (2001) stated that the genomes of *Listeria innocua* and *Listeria monocytogenes* have both been characterized, revealing a close association of both to *Bacillus subtilis*. Glaser et al. (2001) stated that this indicated the three species are very closely related and may have lineage to a common organism. They further hypothesized that *L. monocytogenes* underwent subsequent changes which did not take place in *B. subtilis*, including addition of unique genes that potentially led to increased virulence.

#### Lineage and serovars

Over 90% of *L. monocytogenes* isolates recovered from foods and food processing environments are strains of serogroup 1/2, especially serotypes of 1/2a and

1/2b (Pan, Breidt, & Kathariou, 2009; Gellin & Broome, 1989). O'Connor et al. (2010) evaluated PGFE patterns of a total of 145 *L. monocytogenes* isolates collected from foods and food processing facilities in Ireland between 2004 and 2007. They found that the most common serotype was 1/2a at 57.4%, followed by 4b at 14.1%, 1/2b at 9.7%, and 1/2c at 6.7%. Aarnisalo, Autio, Sjoberg, Lunden, Korkeala, and Suihko (2003) collected a total of 486 *L. monocytogenes* isolates originating from 17 Finnish meat, poultry, fish, and dairy processing facilities and the serotype of each isolate was identified. Serogroup 1/2 was most prevalent (>90%), with isolates of serogroup 4 being the lowest at 3.3%. Pan, Breidt, and Gorski (2010) found that *L. monocytogenes* serotype 1/2a formed biofilms which were of greater density than those formed by serotype 4b and this mechanism may explain some of the reason for the greater prevalence of serotype 1/2a in foods and food processing facilities.

The majority of human cases of illness due to *L. monocytogenes* involve strains of just three serotypes: 1/2a, 1/2b, and 4b (Kathariou, Graves, Buchrieser, Glaser, Siletsky, & Swaminathan, 2006). Despite the fact that serotype 1/2a is found more often than any other serotype in food products and food processing facilities, serotype 4b strains cause the majority of human listeriosis outbreaks (McLauchlin, 1990a; Hayes et al., 1991; Pan et al., 2009; Kathariou et al., 2006; Aarnisalo et al., 2003; Gilbreth, Call, Wallace, Scott, Chen, & Luchansky, 2005; Mead et al., 2006; Revazishvili, Kotetishvili, Stine, Kreger, Morris, & Sulakvelidze, 2004; Wallace et al., 2003; Tresse, Shannon, Pinon, Malle, Vialette, & Midelet-Bourdin, 2007; Pan et al., 2010). Gellin and Broome (1989) reported on detailed data from a 1986 population-based survey by the Center for Disease Control that included six regions of the United States. Of the 161 total isolates reported 53 (33%)



were serotype 4b, 51 (31.5%) were 1/2b, 48 (30%) were 1/2a, six (4%) were 3b, two (1%) were 3a and one (0.5%) was 1/2c.

McLauchlin (1990a) analyzed serotypes of *L. monocytogenes* from 1363 patients in the United Kingdom that were diagnosed with listeriosis. Serovar 4b was the predominant serotype, found in 64% of the cases, while serovar 1/2a was identified in 15% of the cases, serovar 1/2b was observed in 10% of the cases, and serovar 1/2c occurred in 4% of cases. For analysis, McLauchlin (1990a) further grouped patients into one of three categories: 1) pregnancy-associated patients, 2) non-pregnant previously healthy patients, and 3) non-pregnant patients with severe underlying illness. Serovar 4b occurred most often in pregnancy-associated cases (74% of these cases) and at the lowest level in those with underlying illness (still at 53% of these cases). Serovars 1/2a and 1/2b both occurred slightly more often in the non-pregnant cases with severe underlying illness (at 18% and 14%, respectively). Pinner et al. (1992) suggested that the presence of *L. monocytogenes* serotype 4b in a ready-to-eat food appears to increase the risk of the food to cause listeriosis.

Kathariou (2002) reported that most publicized outbreaks over the past twenty years have involved serotype 4b and therefore theorizes that the greater number of serotype 4b in *L. monocytogenes* outbreaks and in clinical cases, compared to its much smaller level of occurrence in food isolates suggests that strains of serotype 4b are more virulent to humans than other strains. De Luca, Zanetti, Fateh-Moghadm, and Stampi (1998) in a survey of a Bologna, Italy sewage treatment plant found a high level of *L. monocytogenes* recovered from activated sludge, with the prevailing serotype being 4b. In a test of French wastewater treatment plants, Paillard et al. (2005) also found that raw

sludge had *Listeria* spp. in 89% of samples, with *L. monocytogenes* being in 52% of samples and the most abundant serotype being 4b/4e. A study in the UK showed, through fecal specimen evaluation, that humans were carriers of *L. monocytogenes* and *L. innocua* with 1-3% of specimens evaluated in June-September being positive, and interestingly, no *Listeria* spp. were detected at any other times of the year (MacGowan, Bowker, McLauchlin, Bennett, & Reeves, 1994).

Grif, Patscheider, Dierich, and Allerberger (2003) conducted a study over a one year period on fecal carriage of *L. monocytogenes*. A total of 868 stool specimens from healthy adults in Austria were evaluated, with 31 testing positive for *Listeria* spp., and 10 being positive for *L. monocytogenes*. The longest duration in any patient was four consecutive samples and none of the volunteers experienced any other illness in conjunction with the positive samples. All *L. monocytogenes* positive samples were serotypes 1/2a and 1/2b. Grif et al. (2003) concluded that random testing of food workers for *L. monocytogenes* would not be a way to prevent carriage of *Listeria* into facilities involved with food manufacturing or preparation due to the sporadic nature of occurrence.

Molecular subtyping studies have suggested that *L. monocytogenes* is composed of three distinct evolutionary lineages that differ in their ability to cause human and animal listeriosis (Piffaretti et al., 1989; Wiedmann, Bruce, Keating, Johnson, McDonough, & Batt, 1997; Gray et al., 2004; Ward, Gorski, Borucki, Mandrell, Hutchins, & Papedis, 2004). Wiedmann, Bruce, et al. (1997) found that lineage I contains all strains that have been associated with foodborne listeriosis in humans (primarily serotypes 1/2b and 4b). Ragon et al. (2008) suggested that serotype 4b has

evolved from serotype 1/2b and therefore serotype 1/2b is likely the original serotype for lineage I. Wiedmann, Bruce, et al. (1997) also found that lineage II contained some strains from human and animal cases of listeriosis, but no strains from human listeriosis epidemics, while lineage III contained no human isolates. Liu (2006) also found that *L. monocytogenes* isolates from sporadic and endemic human listeriosis belong to lineage I or II, whereas animal or environmental isolates mostly belong to lineage III. Jia, Nightingale, Boor, Ho, Wiedmann, and McGann (2007) report that differences in internalin proteins (InlA) are believed to be one of the factors that differ in the three recognized lineages of *L. monocytogenes*. The internalin proteins are present on a number of loci on the *L. monocytogenes* genome and play a role in entry of *L. monocytogenes* into host cells (Dussurget et al., 2004; Kelly, Vespermann, & Bolton, 2009).

#### Virulence and pathogenicity

Casadevall and Pirofski (1999) describe virulence as depending on both the pathogenicity of the infectious organism and the immune status of the host. If the host has a weak immune system then the full virulence of the organism is displayed, often resulting in severe host damage. Evans et al. (2004) reported that the overall understanding of the history, evolution, and virulence of *L. monocytogenes* strains linked to major foodborne disease outbreaks remains limited. Neves, Silva, Roche, Velge, and Brito (2008) evaluated 51 *L. monocytogenes* isolates collected from foods and food processing environments and found, via mouse virulence assays, that all were highly virulent or potentially virulent. Roche, Kerouanton, Minet, Le Monnier, Brisabois, and Velge (2009) evaluated 380 strains of *L. monocytogenes* and found that only ten (2.6%)

were identified as low virulence. McLauchlin (1990a) proposed, from a study of 1363 listeriosis patients in the UK, that there is a clear association between virulence and serological type with serovar 4b being the most virulent and serovars 1/2b and 1/2c being less virulent. Tabouret, Derycke, Audurier, and Poutrel (1991) tested pathogenicity of several clinical and food isolates of *L. monocytogenes* by injecting immunocompromised mice with  $10^4$  cfu of the organism. All twenty-nine of the clinical isolates of *L. monocytogenes* (serotypes 1/2a, 1/2b, and 4b) were pathogenic while thirty-three of forty-two food isolates were pathogenic. Overall, sixty percent of the 1/2a isolates were pathogenic while all of the 4b isolates (10 total) were pathogenic (Tabouret, Derycke, Audurier, & Poutrel, 1991). In a guinea pig feeding study, Roldgaard, Andersen, Hansen, Christensen, and Licht (2009) showed a larger quantity of fecal shedding and increased presence of *L. monocytogenes* in organs in clinical and food isolates whereas a reduced level of virulence was observed in a laboratory strain.

Ragon et al. (2008) proposed that since *L. monocytogenes* is an environmental saprophyte, it does not need to infect animals or humans for survival and propagation, so evolutionary changes *L. monocytogenes* to make it pathogenic to mammals was probably not needed to promote long term survival. The authors theorized that serotype 4b evolved from serotype 1/2b, and suggested that its genetic evolution from 1/2b to 4b lead to the increased virulence potential and an increased ability to cause human outbreaks. Vazquez-Boland, Kuhn, et al. (2001) suggested that the association of serotype 4b and foodborne outbreaks may be due to these strains being able to more readily adapt to its new environment in a human host than strains from serogroup 1/2. Kathariou (2002) pointed out that each infection of *L. monocytogenes* has pathogen-to-host interactions that

may be unique, depending on the *L. monocytogenes* serotype and the immune system of the host. Therefore, pathogens have an opportunity to adapt independently in each host, yielding potential genetic variations, which may not be easily detected (Kathariou, 2002). Sleator, Watson, Hill, and Gahan (2009) pointed out that phenotypic variations may also affect virulence differences between strains. Wiedmann et al. (1998) stated that differences in environmental stress adaptation between different strains may lead to varied survival rates among strains and thus indirectly lead to differences in virulence potential. Ragon et al. (2008) suggested that pathogenicity of *L. monocytogenes* strains may not be a key to their survival and thus some *Listeria* strains may have evolved to lesser levels of pathogenicity to aid their survival.

Norrung and Anderson (2000) used experimental infection of chicken embryos to test virulence of 91 total *L. monocytogenes* strains that included human clinical, animal, and food strains. They found that *L. monocytogenes* strains isolated from human clinical and animals cases were more virulent than strains isolated from other sources. Norrung and Anderson (2000) provided two potential explanations for the difference in virulence between serotypes, including the likelihood that more virulent strains are more likely to cause human infection or secondly that growth of some *L. monocytogenes* strains in a host may cause increased virulence. There have been no major differences detected in the outer cell structure of *Listeria* strains that are virulent compared to those that are non-virulent (Fielder, 1988).

Listeriolysin O (LLO) is a hemolysin used by *L. monocytogenes* during invasion of host that plays a key role in lysis of the phagosome and also is critical in blocking host immune system acidification thus allowing *L. monocytogenes* to obtain entry into the host

cell cytoplasm (Cossart, 1988; Kuhn, Pfeuffer, Greiffenberg, & Goelel, 1999; Lecuit, Sonnenburg, Cossart, & Gordon, 2007; Kuhn et al., 2008; Buchanan, Havelaar, Smith, Whiting, & Julien, 2009). The ability of a *L. monocytogenes* strain to synthesize Listeriolysin O is a key determinant of virulence, since strains lacking LLO will not be able to escape the phagosome and invade the host (Buchanan et al. 2009). Cotter et al. (2008) found that a second listeriolysin - Listeriolysin S - is present in 52% of lineage I *L. monocytogenes* strains tested but was absent from lineage II, lineage III, and non-virulent strains. The authors suggested that this hemolytic peptide aids in *L. monocytogenes* survival and also contributes to virulence when evaluated in a mouse model. They also found a unique peptide cluster within Listeriolysin S that they termed “*Listeria* pathogenicity island 3” (LPI-3), that was found in almost all strains associated with *L. monocytogenes* outbreaks, but not in non-virulent strains.

Indeed, more outbreaks of *L. monocytogenes* have been due to serotype 4b than any other serotype. One of the first known outbreaks, and one of the largest, was reported by Linnan et al. (1988) and was due to contamination in Mexican-style soft cheese in a California production facility in 1985. A total of 142 cases of human listeriosis were reported with ninety-three cases occurring in women or their offspring and forty-nine in non-pregnant adults. There were a total of forty-eight deaths – eighteen non-pregnant adults, twenty fetuses, and ten neonates. Of the forty-nine non-pregnant adults, forty-eight had predisposing conditions, which may have led to compromised immune systems. In evaluating the isolates available for study from this outbreak, 86 of 105 (82%) were serotype 4b and 63 of the 86 (73%) were the same phage type as that isolated from packages of cheese from patient’s refrigerators or from local grocery stores. Linnan et al.

(1988) reported that an investigation of the production facility showed some of the cheese was being produced without pasteurization, as testing of the cheese showed a high level of phosphatase (a heat-labile protein that is significantly reduced with increased time and temperature such as the pasteurization step of fresh milk). All products from the production facility were recalled and the facility was closed. Most patients had consumed soft cheese on several occasions and the median incubation time in these patients with multiple exposures was 35 days, with a range of 1 to 91 days. The four patients who were verified to have single exposure had an average incubation time of 31 days, with a range of 11-70 days. The authors speculated that this variability could be due to dose variation between patients (ingestion load of organisms) or it may be due to immunity differences between hosts. Even though diarrhea is commonly believed to be a symptom of listeriosis, none of the pregnant patients reported diarrhea as part of the early symptoms of the illness. Linnan et al. (1988) concluded that the fact that this cheese was produced in an inadequate manner for several months, but that only 142 illnesses were reported was further evidence that *L. monocytogenes* can be distributed widely in contaminated food products without listeriosis occurring in the vast majority of healthy consumers.

Ho et al. (1986) reported on a cluster of illness in Boston area hospitals during September and October 1979. They reported that twenty-three patients had *Listeria monocytogenes* infection, with isolates from twenty of the patients being serotype 4b. Case patients (those with type 4b *L. monocytogenes* infection) tended to have a high rate of hospital-acquired infection and had received antacids or cimetidine (histamine H<sub>2</sub>-receptor antagonist, such as Tagamet™) before listeriosis onset. Case patients also had

gastrointestinal tract symptoms that began at the same time as fever. Raw vegetables were identified as the most likely source of infection and the authors theorized that *L. monocytogenes* was able to survive ingestion because of gastric acid neutralization and then caused enteritis, which was supported by accompanying abdominal pain, nausea, diarrhea, and fever in case patients. Ho et al. (1986) speculated that the reason not all consumers that ingested the contaminated food became ill was due to differences in virulence, dose level, and variation susceptibility within consumers of the infected food.

Gottlieb et al. (2006) reviewed a *L. monocytogenes* outbreak from July to October 2002 that was caused by a strain that was isolated as serotype 4b. Fifty-four case patients were identified in the outbreak and resulted in eight adult and three fetal deaths. Pinner et al. (1992) tested food samples from refrigerators of patients diagnosed with listeriosis and found that a significant amount (33%) of patient's refrigerators that had *L. monocytogenes* in foods contained the same strain as found in the patient, with the majority of these belonging to serotype 4b.

Mujahid, Pechan, and Wang (2008) examined protein expression of *L. monocytogenes* serotype 4b grown on ready-to-eat sliced turkey at 15°C. Some of the proteins expressed by *L. monocytogenes* grown on turkey meat were those known to be involved in metabolism, cell division, virulence, and stress adaptation. The authors concluded that *L. monocytogenes* needs to upregulate certain proteins in order to grow efficiently on RTE meat compared to growing on nutrient-rich media. Virulence genes were also expressed in the control growth method using Brain Heart Infusion broth and the authors hypothesized it is likely that virulence associated proteins may grow at varying levels under the two growth conditions, but the study did not include a



comparison of *L. monocytogenes* counts in the two types of growth media.

However, not all outbreaks have been due to serotype 4b. Olsen et al. (2005) reported on a 2000 outbreak of listeriosis in New York that included 30 patients, and resulted in four deaths in patients 67-78 years of age, two miscarriages, and three fetal deaths. This outbreak was determined to be caused by serotype 1/2a.

### High Risk Individuals

Vazquez-Boland, Kuhn, et al. (2001) reported that many listeriosis patients have reduced T-cell mediated immunity, including pregnant women, infants, the elderly, and immunocompromised adults with other underlying diseases. The health of patients with listeriosis greatly influences their ability to fight the infection, as most healthy individuals usually survive, while those with compromised immune systems have greater mortality rates of >30-40% (Skogberg et al., 1992). Other researchers have reported that listeriosis-related death or other severe symptoms usually occur in people with predisposing conditions or in susceptible populations including fetuses or the immune-compromised (Perez-Rodriguez, van Asselt, Garcia-Gimeno, Zurera, & Zwietering, 2007; Williams, Castleman, Lee, Mote, & Smith, 2009). As an example of effects on immune-compromised individuals, Fernandez-Sabe et al. (2009) reported that their survey of 25,997 single organ transplant patients identified 30 patients (0.12%) with cases of listeriosis at 15 Spanish transplant centers. Eight of the 30 patients died within 30 days of diagnosis of listeriosis (26.7%).

Buchanan et al. (2009) pointed out that fetuses lack a fully competent immune system and are therefore at a greater risk of infection if *L. monocytogenes* crosses the placenta. Buchanan et al. (2009) also stated that the exact cause of fetal death is

unknown and could be due to maternal reaction to fetal infection, breakdown of the placenta, direct infection of the fetus, or a combination of these factors.

Although most cases of listeriosis involved those with suppressed immune systems, there are also adult patients with no obvious underlying condition that have been infected (Farber & Peterkin, 1991). Other researchers report that listeriosis of otherwise healthy adults often have very short incubation periods and result in gastroenteritis, suggesting ingestion of a very high dose of *L.monocytogenes* (Dalton et al., 1997; Salamina et al., 1996; Ramaswamy et al., 2007). Therefore, *L. monocytogenes* does have the potential to infect otherwise healthy adults and this potential is probably dependent on the bacterial population consumed and the degree of virulence of the *L. monocytogenes* strains (Vazquez-Boland, Kuhn, et al., 2001).

#### Lethal dose and time to infection

A number of studies on *L. monocytogenes* infection on murine (mouse), guinea pig, and monkey models have been conducted to gain a better understanding of the effect of the organism in humans. Czuprynski, Faith, and Steinberg (2002) showed that two serovars of *L. monocytogenes* 4b strains reached greater levels in internal mouse organs and caused more severe damage to liver and spleen than did serotype 1/2a and 1/2b strains after intragastric inoculation with  $10^4$  to  $10^6$  CFU *Listeria*. Cabrita, Correia, Ferreira-Dias, and Brito (2004) showed that lethal dose ( $LD_{50}$ ) for *L. monocytogenes* 1/2a strains was  $9.0 \times 10^4$  to  $7.6 \times 10^5$  cfu/ml<sup>-1</sup> and for 1/2b strains was  $8.4 \times 10^4$  to  $1.7 \times 10^6$  cfu/ml<sup>-1</sup> when evaluated in mice via intraperitoneal injection.

Severino et al. (2007) compared virulence in *L. monocytogenes* strains using a mouse model and found large differences in virulence characteristics, with an epidemic

strain of serovar 4b (CLIP80459) having the greatest virulence with an LD<sub>50</sub> of  $1.7 \times 10^2$  CFU. Fiedler (1988) showed that *L. monocytogenes* strains from serotypes 1/2 and 4 had important structural differences in their cell wall teichoic acid structure that may contribute to differences in survival in hosts. Severino et al. (2007) showed that gene expression was different for genes common to both serotypes 1/2 and 4 and this could be a cause of some virulence differences between these strains.

Conner, Scott, Sumner, and Bernard (1989) observed that virulence of *L. monocytogenes* varies among serotypes. Their test of lethal dose (LD<sub>50</sub>) showed a value of 5-53 cells for all but one of the pathogenic strains of *L. monocytogenes*. Based on the fact that 33% of the serotype 4b strains were found to be non-pathogenic, the authors suggested that the mouse model has limitations in determination of pathogenicity in humans. Lecuit and Cossart (2002) point out that in mice the target of infection for *L. monocytogenes* does not appear to be the brain or fetus, as is the case in humans.

Buchanan et al. (2009) and Sleator et al. (2009) pointed out that mouse dose-response is different than observed in humans due to differences in the E-cadherin receptors between the two species. A study by Lecuit, Dramsi, Gottardi, Fedor-Chaiken, Gumbiner, and Cossart (1999) found that in humans, a proline residue at position 16 of the first extracellular domain for E-cadherin played a critical role in interaction with InlA for human host invasion, but in mice a glutamate residue is found at this position and therefore does not allow InlA-mediated entry of *L. monocytogenes* into mouse tissue. Therefore Lecuit et al. (1999) suggested that use of the mouse model for *L. monocytogenes* invasion using InlA or InlB could not be extrapolated to human *L.*

*monocytogenes* invasion. Lecuit and Cossart (2002) point out that *L. monocytogenes* is not an enteropathogen for mice, which supports their belief that *L. monocytogenes* exhibits species-specific pathogenicity towards its host and this makes the mouse model inappropriate for study of response of internalin functions in human hosts.

Smith et al. (2008) reported on testing of 33 pregnant rhesus monkeys that were infected with *L. monocytogenes*. The authors point out the advantages of the use of monkeys over rodents for dose-response testing is that monkeys can be exposed orally in the same manner as *L. monocytogenes* ingestion in food by humans and all evidence to date suggests that the outcome of the infection is the same as for humans. Smith et al. (2008) found that the LD<sub>50</sub> calculated from their study was about 10<sup>7</sup> CFU, and pointed out their LD<sub>50</sub> value is similar to the FAO-WHO risk assessment (Food and Agriculture Organization of United Nations and World Health Organization, 2004), but is much less than the 10<sup>13</sup>-10<sup>14</sup> CFU based on the exponential dose-response graph published in the FDA *L. monocytogenes* risk assessment (FDA, 2003). Williams, Irvin, Chmielewski, Frank, and Smith (2007) reported that ingestion of 10<sup>7</sup> CFU *L. monocytogenes* by pregnant guinea pigs resulted in about 50% stillbirths. The authors found the guinea pigs that had stillbirths shed *L. monocytogenes* in their feces for a longer period of time than those guinea pigs that did not have stillbirths.

Farber and Peterkin (1991) stated that the minimum number of pathogenic *L. monocytogenes* cells required to cause human illness in either normal or susceptible individuals is not known. Norrung (2000) pointed out, there is no dose response data on human consumption of *L. monocytogenes* and therefore the minimum infective dose for humans cannot be fully determined. Gellin and Broome (1989) also stated out that the

infectious dose of *L. monocytogenes* and the incubation period before being able to get a firm clinical diagnosis are not well known.

Even though human studies on *L. monocytogenes* dose response cannot ethically be conducted, a number of *L. monocytogenes* outbreaks and sporadic infections have provided some insight into the infectious dose required to cause human illness. Graves et al. (2005) reported on their investigation of a 1998 multistate outbreak of listeriosis linked to 108 persons in 24 states. The outbreak strain was identified as serotype 4b with a specific PGFE pattern and this same isolate was identified in samples of opened and unopened frankfurters in patient's refrigerators and in storage at the processing facility in question. The numbers of the outbreak strain of *L. monocytogenes* present in all of the food samples was quantified by MPN and direct plating methods and the outbreak strain was found to be at very low levels (below the minimum quantifiable limit of the three tube MPN method). The authors cautioned that even though the *L. monocytogenes* numbers were very low, because the samples were collected from patient's refrigerators up to several weeks after the onset of illness, the counts in the foods may not represent the number of organisms actually consumed by the patients (Graves et al., 2005).

Mead et al. (2006) reported that the average incubation period for *L. monocytogenes* in a 1998 outbreak was shorter than has been reported for other listeriosis outbreaks. They reported that the median time period between consumption and illness onset for twelve cases involving non-pregnant individuals was five days whereas the four cases associated with pregnancy was 25 days. Mead et al. (2006) also found that in at least one case, an elderly man developed invasive listeriosis with the outbreak strain within 48 hours after eating meat from the implicated food processing facility. Linnan et

al. (1988) had previously reported that the incubation period for *L. monocytogenes* may be as long as several weeks. Farber and Peterkin (1991) showed the incubation periods for some sporadic and outbreak cases of listeriosis to be from less than 24 hours to greater than 35 days. They point out that dose of the organism, immunity of the host, and other viral or bacterial infections of the host can all have an effect on the time from initial exposure to detectable onset of illness. Although there is a variable amount of time to onset of listeriosis symptoms, the incubation period for onset of invasive illness generally averages between twenty and thirty days (Linnan et al., 1998; Riedo et al., 1994). Vazquez-Boland, Kuhn, et al. (2001) commented that *L. monocytogenes* has a long incubation period compared to other foodborne pathogens and reasons are not completely understood, but may include a period of slow infection of the host where clinical symptoms are not immediately detected. Because of the long incubation period in most cases of invasive listeriosis, it is often difficult to confirm *L. monocytogenes* contamination of foods, as the food has often been fully consumed or discarded before clinical onset of listeriosis occurs (Gellin & Broome, 1989).

Pinner et al. (1992) evaluated foods in refrigerators of patients that had been diagnosed with listeriosis. *Listeria monocytogenes* was detected in 79 of 123 listeriosis patient's refrigerators. Of the more than 2000 food specimens collected, *L. monocytogenes* was found in 11% of the foods. There was a match of the serotypes identified in foods with the same serotype identified in the patient as the one causing illness in the patient in 33% of the 79 refrigerators in which *L. monocytogenes* was detected.

Food products associated with listeriosis outbreaks have usually been the types of

products which support growth of *L. monocytogenes* (Norrung, 2000) and ingestion of low levels of *L. monocytogenes* in foods may not pose a health risk even in immunocompromised individuals (Norrung, Andersen, & Schlundt, 1999). Further, Mead et al. (2006) pointed out from their study of a 1998 outbreak of listeriosis that there is a poor correlation between levels of contamination in food samples and the potential risk to public health. Mead et al. (2006) further cautioned that in their study, extremely low levels of contamination of *L. monocytogenes* in foods were linked to a very large and deadly human listeriosis outbreak. Therefore virulence of pathogenic strains, immunity of the hosts, and their interactions likely all have important roles in *L. monocytogenes* infection of individuals (Kathariou, 2002; Vazquez-Boland, Kuhn, et al. 2001).

An opportunity to gain some unique insights on listeriosis infection was provided in a study by Riedo et al. (1994). *L. monocytogenes* of serotype 4b was identified in two pregnant women whose only common exposure was attending the same party. Ten of thirty other party attendees met the case definition with the same serotype 4b isolate being detected in a stool sample of one of the case patients. The incubation periods for illness in the two pregnant women were 19 and 23 days from exposure to clinical listeriosis, with diagnosis from positive blood cultures. One of the women had headaches and muscle pain three days after the party and the other developed diarrhea four days after the party. The women were at week 17 and week 21 of the pregnancies at the time of the party, with the first patient incurring fetal demise at 25 days after the party while the second patient delivered a full-term healthy baby. The authors concluded that the mild illnesses after the party may have represented the initial phases of listeriosis or possibly that an organism other than *L. monocytogenes* was responsible for the mild

gastrointestinal illnesses or may have assisted in infection with *L. monocytogenes*.

Schwartz et al. (1989) had previously suggested that co-infecting organisms along with *L. monocytogenes* may be responsible for some outbreaks of listeriosis. Due to the fact that several other people attended this party and even had the matching outbreak serotype isolated from a stool sample, Riedo et al. (1994) suggested that the findings from their study indicates that a more mild illness may occur in healthy persons who consume foods contaminated with *L. monocytogenes*.

Farber and Peterkin (1991) speculated that there may be a substantial amount of cases of food-borne listeriosis which manifest as only a mild gastrointestinal illness in immunocompetent individuals. Many of these cases have a short incubation period with a greater rate of incidence in immunocompetent adults than invasive listeriosis (Dalton et al., 1997; Salamina et al., 1997). Mead et al. (2006) reported that members of the same family ate deli meat that was later implicated with an outbreak strain of *L. monocytogenes* and three members of the family developed severe gastroenteritis the day after consumption. Kaczmarek and Jones (1989) described a woman who had a case of severe listeriosis but her son contracted only a brief, mild gastrointestinal illness, even though the same serotype 1/2a strain was found in both individuals.

Dalton et al. (1997) reported on outbreak of gastroenteritis caused by *L. monocytogenes* infection of chocolate milk at a picnic resulting in forty-five illnesses among the sixty people who consumed the milk. The average age of those that were ill was 31 and none had any chronic immune deficiency. Among those that became ill, symptoms included diarrhea, fever, and abdominal cramps. The average incubation period was 20 hours from consumption to onset of illness. A sample of milk leftover



from the picnic and a matching sample from the dairy showed *L. monocytogenes* counts of  $10^8$ - $10^9$  CFU/ml and, based on the average 8 ounces consumed, the dose may have been as large as  $2.9 \times 10^{11}$  CFU per person. The serotype from the milk and the patients matched as type 1/2b and post-processing contamination of the milk was identified as the source of *L. monocytogenes* (Dalton et al., 1997).

Salamina et al. (1997) reported an outbreak of gastroenteritis among 18 of 39 persons attending a private party in Italy. All those reporting illness were otherwise healthy and had a median age of 28 years. All reported fever and most had diarrhea and nausea within 3 days of the party with a mean onset of 18 hours for those with gastrointestinal illness. *L. monocytogenes* serotype 1/2b was isolated from two patient samples and from foods from the party, although the rice salad that was the primary food suspected (90% match with those that became ill and 0% for those that did not) was not able to be sampled for *L. monocytogenes*, but was sampled for coliform and had levels of  $10^7$  CFU/g. The rice salad (rice, swiss cheese, vegetables, and hard-boiled eggs) had been held at 27-28°C for 24 hours before the party, so most likely contained very high levels of *L. monocytogenes* and thus the high level of ingestion most likely led to the quick onset of gastrointestinal illness.

Sim et al. (2002) also reported an outbreak of noninvasive gastroenteritis in 31 patients in New Zealand that consumed ready-to-eat meats. The incubation period for onset of illness was approximately 24 hours. The estimated intake of *L. monocytogenes* in ham (21 of those involved) was at about  $10^9$  CFU per person (Sim et al., 2002). This level of organisms may have overwhelmed the immune system of those that were inflicted with gastroenteritis. Farber and Peterkin (1991) stated that the number of cells

required to induce illness will be quite variable because of bacterial strain differences and host susceptibility. Munk and Kaufmann (1988) stated that most healthy adults don't normally contract listeriosis and this may be due to previous development of T-cells from a mild subclinical infection with *Listeria* species or other potential gram-positive bacteria which share the same antigens.

Schmid-Hempel and Frank (2007) proposed a new hypothesis of local or distant action to explain the wide difference of infective dose among pathogens. They proposed that if pathogens act locally, attacking an organ or specific body area, then relatively fewer cells of the pathogen will be required. Work by Dussurget et al. (2004) shows that *L. monocytogenes* exhibits local invasion via intestinal mucosa with the help of bacterial membrane-bound internalin proteins. Schmid-Hempel and Frank (2007) proposed that other pathogens such as *Vibrio cholerae* work via distant action and therefore require a high infective dose.

Norrung (2000) asserted that many consumers probably regularly ingest foods containing low numbers of *L. monocytogenes* without becoming ill. If this is indeed the case, then these people probably have developed some level of memory T-cell immunity (Zenewicz & Shen, 2007). Zenewicz and Shen (2007) conducted a study of innate and adaptive immunity of *L. monocytogenes* in mice due to infection with a sub-lethal dose of the organism. Response by the innate and adaptive immune systems resulted in removal of the pathogen from the host and increased resistance to later exposure of *L. monocytogenes*. Kathariou (2002) stated that subclinical infection by low virulence *L. monocytogenes* strains may confer later resistance to closely related high-virulence strains. However, based on control efforts for *L. monocytogenes* in place since about

1999-2000 in the United States, this regular ingestion of *L. monocytogenes* is probably not taking place at the same level as before these control efforts were started. Could this mean that fewer people will have immunity for *L. monocytogenes* in the future, making control in foods even more vital to prevent widespread outbreaks? Callaway, Harvey, and Nisbet (2006) proposed that having no exposure to low levels of pathogenic organisms may result in an immune system that cannot function normally. The authors state that, for example, in Mexico enteropathogenic *E. coli* is commonly found in food and water, but rarely shows up in clinical settings. They theorize that if an immune system is not exposed to low levels of pathogens, then when an actual pathogen is ingested, its colonization may not be hindered by an effective host immune response. This is clearly an area that needs further research and debate.

Buchanan et al. (2009) and Julien et al. (2009) both discuss the analytical approach of Key Events Dose-Response Framework (KEDRF) intended to shed light on the critical factors that determine response to dose, including variability in such response. Buchanan et al. (2009) used fetal listeriosis as an example within the KEDRF analysis to show how survival of the pathogen can be evaluated at each individual host to pathogen interaction (key event) to determine overall probability of survival. Buchanan et al. (2009) pointed out that some of the key events may have a dose response curve that is linear, while others may be non-linear and this type of evaluation could be used to identify those individual that are most at risk.

#### Method of *L. monocytogenes* invasion of host

After its ingestion via a food product or other means, *L. monocytogenes* survival first depends on the organism's ability to withstand the adverse acidic environment of the

stomach in great enough numbers to later grow in the host (Vazquez-Boland, Kuhn, et al., 2001). The use of antacids or cimetidine treatment has been reported to be a risk factor for listeriosis and allows reduced levels of infective dose required to cause illness (Schlech Chase, & Badley, 1993; Ho et al., 1986; Schuchat et al., 1992). This is an indication that gastric acid can potentially destroy a significant amount of *L. monocytogenes* ingested via contaminated foods (Vazquez-Boland, Kuhn, et al., 2001).

O'Driscoll, Gahan, and Hill (1996) reported that *L. monocytogenes* shows increased tolerance to high acid conditions (as low as pH 3.5) after as little as one hour exposure to mild acidic conditions (pH 5.5) and the ability of *L. monocytogenes* to survive through the stomach and the macrophage phagosome is dependent on this ability to adapt to acidic conditions. Animal models have supported the assumption that numbers of *L. monocytogenes* that survive the gastrointestinal tract are proportional to the numbers ingested (Williams et al., 2007; Smith et al., 2008). O'Driscoll et al. (1996) also stated that stationary-phase *L. monocytogenes* are more resistant to low pH, whereas *L. monocytogenes* in exponential growth phase require adaptive exposure to mild pH conditions and that low pH conditions may permit selection of *L. monocytogenes* mutants with a greater level of acid tolerance and a potentially greater level of virulence.

Buchanan et al. (2009) agreed, as they reported that mechanisms of increased acid resistance in *L. monocytogenes* could be linked to increased virulence. Ferreira, Sue, O'Byrne, and Boor (2003) showed that the stress-responsive alternative sigma factor, Sigma B ( $\sigma^B$ ) is at least partially responsible for the acid tolerance response of *L. monocytogenes* and may aid in its survival when exposed to gastric fluids. Sleator et al. (2009) reported that  $\sigma^B$  is critical to pathogenic capability of *L. monocytogenes* during

infection of the gastrointestinal tract. McGann, Wiedmann, and Boor (2007) reported an interaction between  $\sigma^B$  and PrfA in regulating transcription of virulence genes in *L. monocytogenes*. Other researchers have found that there is increased transcription of  $\sigma^B$ -dependent genes at reduced temperatures thus supporting the role of  $\sigma^B$  as a stress-response alternative sigma factor in *L. monocytogenes* (Becker, Cetin, Hutkins, & Benson 1998; Becker, Evans, Hutkins, & Benson, 2000; Liu, Graham, Bigelow, Morse, & Wilkinson, 2002; McGann, Ivanek, Wiedmann, & Boor, 2007; Wemekamp-Kamphuis, Wouters, de Leeuw, Hain, Chakroborty, & Abee, 2004).

#### Steps involved in internalization of *L. monocytogenes* into the host.

Upon uptake from the gastrointestinal tract, *L. monocytogenes* become engulfed within a phagocytic vacuole (Gaillard, Berche, Frehel, Gouin, & Cossart, 1991) and the vacuole becomes quickly acidified (Beauregard, Lee, Collier, & Swanson, 1997). *L. monocytogenes* is believed to be able to delay phagosome maturation and degradation to allow a longer survival time in the phagosome (Alvarez-Dominguez, Roberts, & Stahl, 1997). *L. monocytogenes* secretes listeriolysin (LLO), a protein that plays a role in destruction of the phagosomal membrane, allowing bacteria to escape into the cytoplasm (Kuhn & Goebel, 2007; Portnoy, Jacks, & Hinrichs, 1988). This membrane destruction is crucial for *L. monocytogenes* survival, as those organisms that remain in the phagosome are killed (Goebel & Kreft, 1997; Kuhn & Goebel, 2007). The presence of LLO is a key indicator of virulence, as *L. monocytogenes* strains that do not express LLO are avirulent (Berche, Gaillard, & Sansonetti, 1987; Tabouret et al. 1991). Once in the cytoplasm, *L. monocytogenes* begins to replicate quickly (Kuhn & Goebel, 2007). *L. monocytogenes* may utilize hexose phosphates from the host cell cytoplasm for growth (Ripio, Brehm,

Lara, Suarez, & Vazquez-Boland, 1997). With the start of cytoplasmic replication, *L. monocytogenes* also begins nucleation of host actin filaments through the action of its surface protein ActA (Ireton & Cossart, 1997). The actin filaments are arranged into a tail which can be used to propel *L. monocytogenes* throughout the cytoplasm (Dabiri, Sanger, Portnoy, & Southwick, 1990; Ireton & Cossart, 1997; Tilney & Portney, 1989). The movement via actin leaves a trail of F-actin in the cytoplasm (Mounier, Ryter, Coquisrondon, & Sansonetti, 1994). Movement is random so that some bacteria reach the cell exterior, push into the membrane, and form finger-like protrusions that can be taken up by neighboring cells (Kuhn & Goebel, 2007; Robbins, Barth, Marquis, de Hostos, Nelson, & Theriot et al., 1999), although the mechanism for this uptake is not completely known (Kuhn et al., 1999). Once *L. monocytogenes* moves to neighboring cells they are engulfed by phagocytosis into a vacuole surrounded by two membranes. They are then lysed by LLO and a different phospholipase C (PC-PLC) is utilized to release *L. monocytogenes* into the cytoplasm of the new host cell and the process can continue to repeat (Ireton & Cossart, 1997; Kuhn & Goebel, 2007). Donnenberg (2000) reported that actin-based intracellular motility of *Listeria monocytogenes* and its ability to spread from cell to cell is similar to that of *Shigella*. Cudmore, Cossart, Griffiths, and Way (1995) suggested that intracellular bacterial pathogens have developed a mechanism to utilize the actin cytoskeleton of the host as a means to accomplish their own spread to adjacent cells.

Adhesion to key host cells is a vital step in the establishment of a pathogen during infection of the host (Bonazzi, Lecuit, & Cossart, 2009). Internalin proteins *inlA* and *inlB* were the first factors identified for mediating *L. monocytogenes* invasion into target cells

and thus play important parts in determining *L. monocytogenes* virulence (Dramsi, Biswas, Maguin, Braun, Mastroeni, & Cossart 1995; Gaillard et al., 1991). Dussurget et al. (2004) suggested additional molecules are also necessary for *L. monocytogenes* adhesion and uptake into cells, proving that there is a very complex association between *L. monocytogenes* and host cells during infection. Gaillard et al. (1991) found that the gene *inlA*, part of an 80 kDa surface protein internalin (*inlA*), was required for *L. monocytogenes* to invade epithelial cells. They also determined that it was part of a gene family that also included *inlB*. Bonazzi et al. (2009) reported that presence of InlA protein can be used as an indicator for virulence in humans, but Nelson et al. (2004) cautioned that several virulence factors for *L. monocytogenes* were identified in genomic sequencing of genes for *L. monocytogenes* with low and high levels of virulence, so the presence of these factors does not give a full indication of level of virulence. Jacquet, Gouin, Jeannel, Cossart, and Rocourt (2002) reported that there are two distinct expressions of InlA in *Listeria* species, one of which is the full length form which is found in *L. monocytogenes* of high virulence, and the second is the truncated form, which is associated with low virulence strains including *Listeria innocua*. Nightingale, Windham, Martin, Yeung, and Wiedmann (2005) found that premature stop codons, associated with the truncated form of InlA were found more often in *L. monocytogenes* strains isolated from foods than from strains involved in cases of human listeriosis. Chen et al. (2011) reported that *L. monocytogenes* with the truncated InlA genetic code could have as much as a 10,000-fold lesser level of virulence compared to full length InlA. Nightingale et al. (2005) and Nightingale et al. (2008) therefore concluded that the premature stop codons and truncated forms of InlA were at least partially responsible for

diminished levels of human virulence in many strains of *L. monocytogenes* found in foods. Ragon et al. (2008) stated that it is believed the full-length InlA was the original form and that truncated, low virulent strains of *Listeria innocua* evolved from the original, giving what is believed to be a unique incidence of evolution from high virulence to a reduced level of virulence in a pathogenic organism. Mengaud, Ohayon, Gounon, Mege, and Cossart (1996) identified E-cadherin as the receptor for internalin (inlA) and proved that it plays a key role in binding and invasion of *L. monocytogenes* into nonphagocytic cells. Dramsi et al. (1995) found that *inlB* gene is required for entry of *L. monocytogenes* into hepatocytes but is not required for entry into intestinal epithelial cells. Shen, Naujokas, Park, and Ireton (2000) found that the membrane receptor Met is required by *L. monocytogenes* for InlB-dependent entry into mammalian cells.

The capability of *L. monocytogenes* to be phagocytosed, and thus remain intracellular, helps the organism to avoid the immune system of the host and therefore to continue to survive and replicate (Tilney & Portney, 1989). Zenewica and Shen (2007) state that antibodies are of limited use for fighting off *L. monocytogenes* infection due to the fact that the organism spreads within the cell and does not move into the extracellular environment. Antibody production against virulence factor LLO could provide some protection by blocking *L. monocytogenes* escape from the phagosome (Edelson & Unanue, 2001) and B-cell antibodies may provide some assistance in reducing *L. monocytogenes* infection into vital organs (Ochsenbein et al., 1999).

Lecuit et al. (1999) and Lecuit et al. (2004) proposed that the mechanism by which *L. monocytogenes* crosses the placenta is invasion of endothelial cells via Inl-A



and E-cadherin interaction. Cells with E-cadherin receptors are associated with invasive listeriosis, suggesting a direct interaction between blood-borne *L. monocytogenes* cells in the pregnant host and uptake by the placental endothelial cells, allowing infection of the fetus (Lecuit et al., 2004). Jia et al. (2007) also found that host E-cadherin receptors must bind with internalin proteins for *L. monocytogenes* to establish in host intestines.

Buchanan et al. (2009) listed the key events that take place in the maternal host during listeriosis leading to fetal listeriosis. They include: 1) survival of *L. monocytogenes* through the upper gastrointestinal tract, 2) establishment of infectious levels in the intestine and attachment/uptake into epithelial cells, 3) survival and escape from phagosomes in enterocytes and transfer to cellular phagocytes, 4) transfer of *L. monocytogenes* across the placental barrier, and 5) *L. monocytogenes* growth in fetus, potentially leading to disease or death of the fetus. After infection of the host by *L. monocytogenes*, both innate and adaptive immunity are needed to control the spread of infection within the host (Zenewicz & Shen, 2007).

Glaser et al. (2001) pointed out that the best characterized regulatory factor of *L. monocytogenes* is PrfA, which activates most of the known virulence genes and is absent from *Listeria innocua*. Xayarath, Volz, Smart, and Freitag (2011) reported that PrfA induces gene expression for proteins that initiate spread of *L. monocytogenes* to nearby cells and thus increases virulence. Genomic sequencing of *L. monocytogenes* has shown more information on the potential role of surface proteins. Cabanes, Dehoux, Dussurget, Frangeul, and Cossart (2002) showed that the surface protein LPXTG, which is covalently linked to the peptidoglycan, is often preceded in the gene by a PrfA box, strongly suggesting that these LPXTG proteins are involved in virulence expression. If a

mutated protein is substituted for virulent PrfA protein in the PrfA box region, the resulting *L. monocytogenes* was found to not be virulent due to virulence genes not being turned on by the mutant protein (Velge et al. 2007). Some of the known virulence genes of *L. monocytogenes* are clustered on the chromosome in the PrfA-dependent virulence gene cluster (Kuhn & Goebel, 2007; Portnoy, Chakraborty, Goebel, & Cossart, 1992). The cluster has been termed *Listeria* pathogenicity island 1 (LAPI-1) and comprises six virulence factor genes: *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB* (Vazquez-Boland, Dominguez-Bernal, Gonzalez-Zorn, Kreft, & Goebel, 2001). The internalin proteins internalin A (InlA) and InlB are encoded by the InlAB operon (Kuhn & Goebel, 2007). Chen, Kim, Jung, and Silva (2008) showed that expression of both InlA and InlB plays a key role in strength of attachment of *L. monocytogenes* to surfaces and thus they may be important for adherence to food equipment surfaces and increase the chance of becoming transferred to foods items during manufacturing.

Evans et al. (2004) reported that a unique strain of *L. monocytogenes* serotype 4b that they termed Epidemic Clone II (ECII) was discovered during an outbreak in hot dogs in 1998 to 1999. They further reported that this strain has unique regions adjacent to the internalin genes *inlA* and *inlB* suggesting that these genes may be uniquely involved in interactions of this strain with the host and may confer a greater level of virulence in this unique strain. Kathariou et al. (2006) pointed out that the ECII isolates show a unique diversification in the genome sequence “region 18” that has been conserved on all other serotype 4b isolates of *L. monocytogenes*. Kathariou et al. (2006) also noted that ECII was a very rare strain within PulseNet database prior to 1998, but has been found much more often since that time, which they speculated may be due to a yet unknown source of

this strain that has potentially led to its introduction within food processing facilities.

These studies collectively show that *L. monocytogenes* is able to adjust and adapt to its environment to aid in its survival. In most cases this adaptation takes place gradually over a number of minutes to potentially hours. This ability to adapt is a complex process that requires interaction of genes, proteins, regulatory factors, membrane permeability, and potentially the formation of biofilm layers in groups of cells (Gandhi & Chikindas, 2007). Therefore, the addition of materials into recipes, or use of processes that are more immediate and do not allow *L. monocytogenes* time to adapt to the stress, should result in a greater level of *L. monocytogenes* inactivation and a reduced potential for repair and regrowth of injured cells.

### **Foods at risk**

There are a significant number of ready-to-eat foods that are at risk for *L. monocytogenes* contamination. Gombas, Chen, Clavero, and Scott (2003) found in their analysis of products collected at retail establishments from eight ready-to-eat food categories, that at least a few samples from all categories were positive for *L. monocytogenes*. The categories included soft cheeses, bagged salads, blue-veined cheese, mold-ripened cheese, seafood salads, smoked seafood, luncheon meats, and deli salads. Contamination incidences ranged from an average of 0.17% for fresh soft cheeses up to 4.70% for seafood salads. Luncheon meats showed a fairly low average contamination incidence, but was more apt to have a greater level of *L. monocytogenes* contamination ( $>10^2$  CFU/g) in manufacturer packaged items.

Pradhan et al. (2010) showed that there is significant risk for listeriosis-associated deaths caused by ready-to-eat meat products and that the risks increase with increased

handling of the product (via retail delis) as well as with increased storage temperatures.

Lin et al. (2006) determined that *L. monocytogenes* coming into contact with a commercial slicer blade, or other parts that contact the meat surface, could subsequently be transferred onto the surface of sliced meats. Their data showed a strong correlation between the level of *L. monocytogenes* on the slicer blade and the amount transferred to the meat surface. This potential contamination and attachment of *L. monocytogenes* to equipment surfaces could lead to contamination of subsequently sliced products at retail establishments or at larger-scale manufacturing facilities.

Seman, Borger, Meyer, Hall, and Milkowski (2002) found that increasing amounts of finished product moisture can increase the growth rate of *L. monocytogenes*. Other researchers have shown that sodium nitrite (Buchanan, Stahl, & Whiting, 1989; Buchanan & Phillips, 1990; McClure, Kelly, & Roberts, 1991; Schlyter, Glass, Loeffelholz, Degnan, & Luchansky, 1993; Grau & Vanderlinde 1992; Duffy, Vanderlinde, & Grau, 1994; Farber & Daley, 1994b; Vitas, Aguado, & Garcia-Jalon, 2004) and sodium chloride (Glass & Doyle 1989; McClure et al. 1991; Seman et al., 2002; Legan, Seman, Milkowski, Hirschey, & Vandeven, 2004) can influence the growth rate of *Listeria monocytogenes*.

Turkey has been shown in a number of studies to be an excellent growth media for *L. monocytogenes* (Lianou, Geornaras, Kendall, Scanga, & Sofos, 2007; Ojeniyi, Christensen, & Bisgaard, 2000) and can grow to high levels in a short period of time (Pradhan, Ivanek, Grohn, Geornaras, Sofos, & Wiedmann, 2009; Burnett, Mertz, Bennie, Ford, & Starobin, 2005).

**Effects of temperature, pH, and water activity on growth rate of *Listeria***

### ***monocytogenes* in foods**

*L. monocytogenes* has a growth range of 0-45°C (Lado & Yousef, 2007) and an optimum growth temperature of 37°C according to USDA-ERRC growth model (Eastern Regional Research Center, 2011). Growth of *L. monocytogenes* is reduced but not prevented at refrigerated temperatures of 0-5°C, making it a pathogen of great concern for refrigerated food products (Kathiarou, 2002; Zhu, Du, Cordray, & Ahn, 2005; Lado & Yousef, 2007). It is also salt tolerant and can grow in the presence of salt at refrigerated temperatures (Lou & Yousef, 1999). Grau and Vanderlinde (1992) showed that *L. monocytogenes* grew at slow rates at 0.1°C, but as storage temperature increased up to 15°C, *L. monocytogenes* grew much faster than other organisms. The growth rate of *Listeria monocytogenes* was modeled by Hwang and Tamplin (2007) and they found an increase in storage temperature from 0°C to 36°C increased the growth rate of *L. monocytogenes* in ham at a linear rate. Pradhan et al. (2010) modeled several intrinsic and extrinsic factors for *L. monocytogenes* growth and determined that storage temperature had the most significant impact on growth of the organism from production to retail sale, and also had the greatest impact on potential number of listeriosis deaths from retail to consumption. The authors cited an example in the elderly population that the baseline of 13.2 deaths per year would be increased to 27.2 with a 3°C increase in storage temperature. Based on their estimate, up to 41% of the estimated deaths due to *L. monocytogenes* could be caused by home refrigerators with a temperature above 10°C (Pradhan et al., 2010). Fortunately, the average temperature for refrigerated storage of food items has decreased over the past few years. In a study by EcoSure (Ecolab, 2008), the average temperature for home refrigerators in the study was 3.4°C (38.2°F), with 17%

of the total home refrigerators above 5°C (41°F), 5% above 7°C (45°F), and 0.5% above 10°C (50°F). This is an improvement over previous data from Audits International (1999) when the average temperature for home refrigerators was 4.0°C (39.2°F) and about 27% of home refrigerators had a temperature above 5°C (41°F), 10% above 7°C (45°F) and 2% were above 10°C (50°F). The most recent survey showed the average retail display cooler temperatures for all coolers was 4.4°C (40.0°F) with 31% of the total display coolers above 5°C (41°F) (Ecolab, 2008), which again was an improvement from the 1999 survey which had an average temperature for all refrigerated cases of 41.7°F (Audits International, 1999). The 2008 survey showed that the pre-packaged lunch meat, service deli, and pre-packaged deli display coolers averaged 5.2°C (41.3°F), 6.4°C (43.6°F), and 4.4°C (39.9°F), with 38%, 58%, and 31% above 5°C (41°F), respectively (Ecolab, 2008). In the earlier survey these coolers were at 6.4°C (43.6°F), 7.1°C (44.8°F), and 5.7°C (42.3°F), respectively with 60%, 71%, and 54%, respectively above 5°C (Audits International, 1999). In 2008, the average backroom refrigerated storage temperature at retail was 2.2°C (35.9°F) with 9% of these at greater than 5°C (41°F) (Ecolab, 2008), while in 1999 backroom storage had averaged 3.3°C (37.9°F) with 17% above 5°C (Audits International, 1999).

Norrung (2000) reported that *L. monocytogenes* can grow at pH values of 4.5-9.2 and at water activity levels >0.92, while Parish and Higgins (1989) found that *L. monocytogenes* could grow in tryptic soy broth at pH values from 4.5 to 7.0, but failed to grow at greater than pH 7.

#### **Prevalence of *Listeria monocytogenes* in RTE foods in 1980's and 1990's.**

A number of studies were conducted in the 1980's and early 1990's showing that

*Listeria* spp., and in many cases *Listeria monocytogenes*, specifically were present in a significant percentage of ready-to-eat food products. Farber and Daley (1994b) collected a total of 101 paté samples representing 25 different types of patés from retail locations in Ottawa, Ontario, Canada and evaluated for presence of *Listeria* species. Of the 101 samples, 21 tested positive for *Listeria* with seven of these testing positive for *L. monocytogenes*. Very low numbers of *Listeria* (<10 cfu/g) were found on products and although *L. monocytogenes* was able to survive over the 3 week sampling period, it only grew on one sample (turkey paté) when held at 4°C. When paté samples were inoculated with *L. monocytogenes* ( $10^3$  cfu/g), there were not increased numbers of the organism after 21 days. Other products obtained during random sampling (vacuum packaged sliced ham, turkey breast, and wieners) were found to be naturally-contaminated with *L. monocytogenes* at low levels (<10 cfu/g). *L. monocytogenes* was able to survive over a 4 week storage period, but either stayed at the low level or grew slowly at 4°C. The sliced ham products had Lactobacilli counts of  $4.8 \times 10^7$  to  $8.6 \times 10^7$  by the end of the 4 week storage period and the authors concluded that these high counts of spoilage organisms may have inhibited the growth of *L. monocytogenes* in the product.

Farber, Sanders, and Johnston (1989) collected a total of 744 samples of food products from Canadian retail establishments and evaluated for the presence of *L. monocytogenes*. As would be expected, *L. monocytogenes* was found in a large percentage of raw meats items (37 of 60), but was also found in 20% (6 of 30) of fermented sausage items and in <1% (2 of 530) of ice cream samples.

Grau and Vanderlinde (1992) sampled commercial products (corned beef, ham, and fermented salami) from Australia for the presence of *Listeria*. *Listeria* species was

detected in 53% of product collected, with *L. monocytogenes* detected in 45% of products. MacGowan et al. (1994) conducted a year-long sampling of *L. monocytogenes* in various locations in Bristol, England (UK). They found *L. monocytogenes* in 10.5% of food items, 60% of sewage samples, and 0.7% of soil samples during the sampling period. No food items were found to contain levels of *L. monocytogenes* greater than  $10^4$  CFU/g.

Wang and Muriana (1994) evaluated frankfurters obtained at retail supermarkets for *Listeria* spp. and *L. monocytogenes*. *Listeria* spp. was found at an incidence of 10% (9 of 93 packages) and *L. monocytogenes* was found in 7.5% (7 of 93 packages). *L. monocytogenes* was not found in any of the frankfurters, but only in the exudate of the packages, which suggests that this was due to post-lethality contamination. The levels of *L. monocytogenes* were low, ranging from none detected up to 27.6 MPN per package. Wang and Muriana (1994) used the 3 tube MPN method with  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  dilutions being used. Therefore the levels of *L. monocytogenes* were approximately  $<2 \log$  CFU/gram.

PulseNet is a national network established in 1996 for the purpose of aiding public health agencies and the CDC in quickly identifying any patterns of illness caused by foodborne pathogens (Swaminathan et al., 2001). The system utilizes analysis and exchange of DNA fingerprints to identify any clusters of illnesses that may be caused by a single strain of a given foodborne pathogen. A pulsed-field gel electrophoresis (PGFE) analysis is used to provide a fingerprint of a given strain of foodborne pathogens that can then be compared to other local and national databases to identify other geographic areas where this strain has been detected (Halpin, Garrett, Ribot, Graves, & Cooper, 2010).



Graves et al. (2005) described how the state of New York was able to contribute *Listeria* PGFE patterns from an outbreak in 1998 and how the source of the outbreak may have been identified more quickly if more labs had been trained in the protocol. As a result of this and other outbreaks, protocols were quickly put in place for PGFE procedures for identifying specific strains that could then be posted on PulseNet for other groups to view. Graves and Swaminathan (2001) describe the protocol for *Listeria monocytogenes* subtyping that was added to PulseNet evaluation in 1999, in addition to *E. coli* O157:H7, non-typhoidal *Salmonella*, and *Shigella* isolates that were already being evaluated. Halpin et al. (2010) described further changes to the PGFE protocol for *L. monocytogenes*, implemented in 2006, to improve the time required, the quality of results, and the cost of the PGFE procedure.

#### **Level of *L. monocytogenes* in recent years in meats**

In a more recent report, Wallace et al. (2003) conducted a study over a two year period to determine the prevalence and identify types of *L. monocytogenes* in vacuum-sealed frankfurters obtained from 12 commercial manufacturers and determine if the presence/level could be correlated with formulation, temperature, and/or storage time. *L. monocytogenes* was recovered from 543 of 32,800 (1.66%) packages of frankfurters. The recovery rate was not influenced by the age of product (time of evaluation after manufacturing) or by the two different storage temperatures (4°C or 10°C). The study did not conduct tests for enumeration of organisms, but frozen samples from 157 positive packages were tested and, other than three packages from one plant at 71, 95, and 191 MPN, all packages tested negative. Over 90% of the positive samples were of serotype 1/2a. The authors also reported USDA/FSIS data that showed after intact packages

initially testing negative for *L. monocytogenes* were held for an additional 6 weeks at 40°F, it was possible to isolate *L. monocytogenes* from 18 of 1984 (0.91%) samples (Wallace et al. 2003).

Gombas et al. (2003) found in their survey of retail luncheon meats conducted in 2000 and 2001 that 2.7% of ready-to-eat deli meats handled at retail were positive for *L. monocytogenes*, whereas 0.4% of deli meats packaged at manufacturers were positive for *L. monocytogenes*. The contamination levels of 99.4% of all samples were less than one CFU/g of *L. monocytogenes*.

Sauders et al. (2009) conducted a survey of 121 retail food establishments regulated by the New York State Department of Agriculture and Markets, collecting food and environmental samples to evaluate for presence of *L. monocytogenes*. *L. monocytogenes* was detected in 2.7% of food samples, 13.0% of environmental samples, and at least one positive *L. monocytogenes* sample (food and/or environmental) was obtained from 60.3% of establishments throughout the 12 month sampling period (2005-2006). Sauders et al. (2009) found the same *L. monocytogenes* strains in various locations in the same establishment and therefore concluded that cross-contamination from one portion of the establishment to another was occurring.

## ***Listeria* Control Efforts**

### **Routes of post-lethality contamination**

Raw meat materials have the capability of supporting the survival and growth of *L. monocytogenes* (Wimpfheimer, Altman, & Hotchkiss, 1990; Chasseignaux, Toquin, Ragimbeau, Salvat, Colin, & Ermel, 2001; Doi, Ono, Saitoh, Ohtsuka, Shibata, &

Masaki, 2003; Yucel, Citak, & Gundogan, 2004; Doijad et al., 2010; Pesavento, Ducci, Nieri, Comodo, & Lo Nostro, 2010). The heat processing methods used by manufacturers of processed meats in the United States are sufficient to eliminate *L. monocytogenes* from ready-to-eat products, but recontamination may occur at several handling steps after the lethality cook, including slicing and packaging (Hwang & Tamplin, 2007; Kornacki & Gurtler, 2007). Vorst, Todd, and Ryser (2006) stated that machinery such as slicers have the ability to harbor pathogens such as *L. monocytogenes* and then transfer the pathogens to items sliced at a later point, and if these items support the growth of the pathogen they could pose a risk to consumers of these items, especially consumers that are immunocompromised. In fact, an outbreak of listeriosis in 1998 that caused approximately 108 illnesses and 18 deaths was traced to post-lethality contamination at a commercial processing facility (CDC, 1999; Graves et al., 2005; Mead et al., 2006).

### **USDA Directive 10,240 for processing Alternatives 1, 2, or 3**

In May 1999 (FSIS, 1999), based on some of the recalls and outbreaks caused by *L. monocytogenes* in meat and poultry products, USDA-FSIS required manufacturers of ready-to-eat products to reassess their Hazard Analysis Critical Control Point (HACCP) plans to assure that they were adequately addressing the risk of *L. monocytogenes* in their facility. If the reassessment showed that *L. monocytogenes* was a hazard “reasonably likely to occur”, then changes to the plant’s HACCP plan needed to be made to address this risk.

USDA-FSIS published new regulations in June, 2003 (FSIS, 2003) intended to reduce the risk of contamination of ready-to-eat meat and poultry products by *Listeria monocytogenes*. Processors were allowed to use one of three alternatives to address the

control of *L. monocytogenes* in their products. “Alternative 3” items were defined as those that controlled *L. monocytogenes* by use of sanitation and cleaning only.

“Alternative 2” items were defined as those that controlled *L. monocytogenes* by use of either antimicrobial agents or by a use of a post-lethality intervention treatment.

“Alternative 1” items were defined as those that controlled *L. monocytogenes* by use of both an antimicrobial agent and a post-lethality intervention treatment. The post-lethality intervention treatments used for either Alternative 1 or 2 products must be addressed by the plant’s HACCP plan and the treatment must be a critical control point (CCP) for the HACCP plan.

#### **Prevention via cleaning and sanitation practices.**

Initial control efforts for *L. monocytogenes* centered on improvements in cleaning and sanitation practices. Hugas et al. (2002) listed some of the methods used to reduce cross-contamination in ready-to-eat meats, such as cooked ham, including improved sanitation practices, post-cook pasteurization, and even the use of ultra-clean facilities such as “white rooms” for slicing and packaging.

Berrang, Meinersmann, Frank, and Ladely (2010) described the evaluation of *L. monocytogenes* shortly after construction of a new chicken further-processing facility. The constructed plant received cut-up raw boneless parts from detached slaughter plants at several locations. Prior to any meat being processed in the plant, all floor drains were negative for *L. monocytogenes*. Within one month, *L. monocytogenes* was isolated from drains before cleanup and within 5 months, *L. monocytogenes* was detected in a drain after completion of cleaning and sanitizing. No *L. monocytogenes* was found on floor swabs or on air filters, so the authors were not able to demonstrate entry of this organism

into the plant via personnel or fresh air intake. The authors concluded that the presence of a persistent subtype of *Listeria monocytogenes* in a facility could increase the likelihood of transfer of this organism to cooked, ready-to-eat product (Berrang et al. 2010).

Takahashi, Kuramoto, Miya, and Kimura (2011) tested survival of inoculated *L. monocytogenes* isolates on stainless steel with or without food component soil on the stainless steel. Up to 3 log CFU of *L. monocytogenes* remained on the stainless steel after dehydrated storage for 30 days. The authors concluded that under the right conditions, *L. monocytogenes* could colonize in food processing facilities and potentially cause contamination of foods for long periods of time (Takahashi et al., 2011). Kalmokoff, Austin, Wan, Sanders, Banerjee, and Farber (2001) evaluated adsorption of 36 *L. monocytogenes* isolates and found no correlation between attachment to type 304 stainless steel and the *L. monocytogenes* serotype or the source of the organism. They found that there was increased attachment to the stainless steel surface when strains produced extracellular fibrils, although only one of the strains produced a biofilm. Park, Haines, and Abu-Lail (2009) further evaluated adhesion of *L. monocytogenes* to metal surfaces and found stronger adhesion of virulent strains. The authors concluded that these adhesive properties may provide for increased environmental survival and therefore increase the possibility of infecting animals or humans. Cabanes et al. (2002) concluded that strains of *L. monocytogenes* have a variety of mechanisms for survival including numerous surface proteins and several methods for anchoring to a variety of surfaces which aid in their survival and growth in a wide range of environments.

So, clearly *L. monocytogenes* have the ability to survive, and potentially grow, in

food processing environments, and it is therefore not enough to depend solely on cleaning and sanitation practices, but additional safeguards need to be in place to assure control of *L. monocytogenes* in ready-to-eat products.

### **Effects of typical curing ingredients on *L. monocytogenes* growth**

Glass and Doyle (1989) procured several processed meat products in retail packages from processors and then inoculated each with a five-strain mixed culture of *Listeria monocytogenes* at approximately  $10^5$  cells per package and evaluated at up to twelve weeks of storage. Meats tested included sliced turkey, sliced chicken, ham, bologna, wieners, fermented semidried sausage, bratwurst, and cooked roast beef. Growth of the organism on processed meats was closely related to the pH of the product. The organism grew well on meats near pH 6 and above but grew poorly or not at all on products close to or below pH 5. Of the two different sliced turkey products that were tested, one formulation that had a greater concentration of salt (sodium chloride at 2.7%) and carbohydrate had less growth than the sliced turkey with a lesser concentration of salt (sodium chloride at 1.3%). The product with greater level of carbohydrate/salt had a more rapid pH decline, which the authors attributed to the lactic acid bacteria fermentation of the available carbohydrate. This pH drop via lactic acid bacterial fermentation, in addition to the greater salt level may have led to some inhibition of *L. monocytogenes* growth through the latter portion of the shelf life study.

Grau and Vanderlinde (1992) showed that *L. monocytogenes* grew more slowly on hams with the greatest residual nitrite concentrations, thus showing that residual nitrite may also influence the rate of growth of *L. monocytogenes* in refrigerated meats. They also showed that refrigerated meats with high pH and high  $a_w$  may allow greater growth

of *L. monocytogenes* during product shelf life. McClure et al. (1991) tested growth of *L. monocytogenes* in tryptone soya broth and found some inhibition from sodium nitrite especially at greater levels of addition and at lesser pH levels (< 6.0).

A study by Schlyter et al. (1993) evaluated growth of *L. monocytogenes* in uncured ready-to-eat turkey meat. Commercial cured turkey breast was obtained to compare nitrite level by pasteurizing product (68°C/10 minutes) and then adding 30 ppm of sodium nitrite to the product after cooking and before inoculation, to simulate the estimated nitrite level remaining in product after lethality cook. The tests revealed that uncured turkey breast meat provided an excellent growth environment for *L. monocytogenes*. They also found that sodium nitrite alone at 30 ppm did not inhibit growth of *L. monocytogenes*. The authors concluded that low concentrations of nitrite (such as the 30 ppm used in their experiment) are insufficient to inhibit growth of *L. monocytogenes*.

Buchanan et al. (1989) tested combinations of sodium nitrite, storage temperature, pH, packaging atmosphere, and sodium chloride. They found the combination of greater concentrations of sodium nitrite along with reduced temperature (<5°C) and pH (6.0) gave the greatest bacteriostatic effect for *L. monocytogenes*.

Lianou et al. (2007) studied commercial uncured turkey breast items inoculated with *L. monocytogenes* over a fifty day shelf life and witnessed steady growth of the pathogen. The authors concluded that this study provided further evidence that *L. monocytogenes* can grow to high levels in uncured products and *L. monocytogenes* growth may be inhibited by sodium nitrite, which was also found by other authors (Duffy et al., 1994; Farber & Daley, 1994b; Vitas et al., 2004). Lianou et al. (2007) also

suggested that in evaluation of samples later in shelf life (25 and 50 days), high levels of lactic acid spoilage organisms were encountered and may have contributed to lack of further growth of *L. monocytogenes* during subsequent aerobic storage. Samelis, Kakouri, and Rementzis (2000) commented that typical processed meat conditions favor the growth of psychrotrophic lactic acid bacteria.

### **Antimicrobial ingredient addition**

Research has been conducted on several ingredients to control growth of *L. monocytogenes*. Hwang and Tamplin (2007) modeled growth of *L. monocytogenes* in ground ham with and without addition of sodium lactate and/or sodium diacetate. They modeled lag phase duration and found that a decrease in storage temperature or an increase in the level of lactate or diacetate increased the lag phase duration of *L. monocytogenes*. In addition to lag phase duration, Hwang and Tamplin (2007) also modeled the growth rate of *L. monocytogenes* and found that an increase in storage temperature increased the growth rate of *L. monocytogenes* in ham. At 4°C and 8°C, increased levels of diacetate slightly increased growth rate of *L. monocytogenes* and an increase in the level of lactate had little effect on growth rate.

Lianou et al. (2007) studied commercial turkey breast with and without antimicrobial ingredients potassium lactate and sodium diacetate. They found that turkey breast containing lactate and diacetate combinations still showed significant growth during the storage period and the authors concluded that levels of antimicrobial ingredients in some formulation were not adequate to prevent growth of *L. monocytogenes* to greater levels. Wederquist, Sofos, and Schmidt (1994) found that addition of antimicrobial ingredients inhibited growth of *L. monocytogenes* over a 98 day



storage period compared to a control product which grew to  $> 9$  log CFU/g from the initial inoculation of  $\sim 2.5$  CFU/g. Sodium acetate gave the greatest inhibition of *L. monocytogenes* with counts at 1.33 log CFU/g at the end of storage, followed by potassium sorbate and sodium lactate which had levels of 2.13 and 2.51 log CFU/g at the end of storage, respectively.

Seman et al. (2002) developed a mathematical model capable of predicting the growth of *L. monocytogenes* in commercial cured meat products. They concluded that increasing amounts of sodium diacetate and potassium lactate syrup resulted in a decrease in *L. monocytogenes* growth rate. Sodium chloride was not a significant factor in the study ( $P>0.30$ ), but had a negative correlation coefficient.

Legan et al. (2004) used a central composite response surface design to predict growth of *L. monocytogenes* as a function of five variables: sodium chloride, sodium diacetate, potassium lactate, cured/no cure, and finished product moisture in ready-to-eat cured meat products. Their model showed that as salt, diacetate, lactate, and cure levels increased and as moisture level decreased, there was decreased growth of *L. monocytogenes*. Validation data for the model was also presented and supported the model well for all cured items, but for uncured items was not validated at low salt and high moisture levels.

Byelashov, Carlson, Geornaras, Kendall, Scanga, and Sofos (2009) tested *L. monocytogenes* survival in pepperoni with a pH of 4.67 and a water activity of 0.827. The initial inoculation level was at 3.0-4.0 log CFU/cm<sup>2</sup>. At 4°C, *L. monocytogenes* counts declined steadily by approximately 1.5 log in the first 4 days, then by 0.022-0.110 log/day for the next 26 days. Counts declined more rapidly at 12°C and 25°C, decreasing

by 2.5 and 4.0 logs, respectively, by day 4. *L. monocytogenes* was not detected after day 60 through day 180 days of storage regardless of inoculum type and storage temperature. The presence of high levels of organic acids in the fermented pepperoni may have led to the increased rate of decline of *L. monocytogenes* at greater temperatures (Byelashov et al., 2009). Also, undissociated molecules of acids may disrupt the proton-motive force (PMF) of cells, which can lead to cell death as most of the adenosine triphosphate (ATP) from the cell is expended in pumping protons out of the cell to maintain pH equilibrium (Brul & Coote, 1999). Abou-Zeid, Oscar, Schwarz, Hashem, Whiting, and Yoon (2009) tested growth of *L. monocytogenes* at various temperature, pH and potassium lactate/sodium diacetate concentrations and showed that *L. monocytogenes* did not grow well at low pH (5.5) or at increased concentration (3.0%) of potassium lactate/sodium diacetate.

Seman, Quickert, Borger, and Meyer (2008) further studied the effects of various concentrations of salt, sodium diacetate, sodium benzoate, and finished product moisture on growth of *L. monocytogenes*. They found that increasing levels of salt, sodium diacetate, and sodium benzoate all increased the time to growth for *L. monocytogenes*. They also found that benzoate and diacetate were not as effective in preventing growth in increased moisture products (>60%) compared to their effectiveness in reduced moisture items.

Sivarooban, Hettikrachchy, and Johnson (2008) investigated the changes in *L. monocytogenes* growth when treated with nisin and grape seed extract (GSE), green tea extract (GTE), or their purified phenolic compounds. When evaluated by transmission electron microscopy (TEM), control cells exhibited smooth membranes, but cells treated

with phenolics, GSE, or GTE had less structural integrity with the cell surface being very disordered and the cytoplasm showing some aggregation. The authors believed that the ability of nisin to form pores into the cell increased the rate of diffusion of the phenolic compounds into the cell.

Chung, Vurma, Turek, Chism, and Yousef (2005) showed that strains of *L. monocytogenes* had different sensitivities to nisin. Those that were sensitive to nisin showed this sensitivity at 62.5 to 100 IU/ml and those that were more resistant showed some sensitivity at 500 to 2000 IU/ml. Abee, Krockel, and Hill (1995) reported that the target site of nisin's antimicrobial action is the cytoplasmic membrane.

Jofre, Garriga, and Aymerich (2007) tested antimicrobial addition to interleaving paper and found the combination of nisin and lactate was most effective. In comparing the results of their study using nisin on interleavers with a similar study of antimicrobial application via formulation by Aymerich, Jofre, Garriga, and Hugas (2005), the authors concluded that the increased efficacy of nisin when applied through interleavers is likely due to its localization on the surface of the slices where the contamination of *L. monocytogenes* is also likely occurring.

A study by Burnett, Chopskie, Podtburg, Gutzmann, Gilbreth, and Bodnaruk (2007) documented the use of octanoic acid to reduce *L. monocytogenes* in ready-to-eat meats. Octanoic acid was applied to the surface of ready-to-eat meats after a lethality cook process had been applied and product had been cooled. After treatment with a solution of 1% octanoic acid at a level of 1.9 ml per 100 cm<sup>2</sup>, the packages were then vacuum-sealed and exposed to a shrink tunnel for ca. 2 seconds or 7 seconds. Addition of octanoic acid decreased *L. monocytogenes* counts in oven roasted turkey by 2.63-2.83

log CFU in the 2 second shrink tunnel exposure and 2.85-2.99 log CFU in the 7 second exposure. In cured ham, octanoic acid decreased *L. monocytogenes* counts by 2.71-2.89 log CFU in the 2 second shrink tunnel exposure and 3.19-3.34 log CFU in the 7 second exposure. The study also included sensory evaluation, which showed no significant differences between the treated and untreated turkey and ham items. The authors theorized that the mechanism for *L. monocytogenes* damage was the combination of octanoic acid and a short heat cycle that weakened the organism's cytoplasmic membrane, allowing octanoic acid to penetrate into the cell (Burnett et al., 2007).

Other researchers (Luchansky, Call, Hristova, Rumery, Yoder, & Oser, 2005; Stopforth, Visser, Zumbrink, Van Dijk, & Bontenbal, 2010) have evaluated the use of lauric arginate as a surface treatment to control the growth of *L. monocytogenes*. Luchansky et al. (2005) showed that hams that were surfaced treated with a 5% solution of lauric arginate had *L. monocytogenes* reductions of 5.1-5.5 log CFU/ham. Over a 60 day shelf life, though, *L. monocytogenes* was able to repair and grow, with the level of growth being 2.0 log CFU/ham in the high level (8 ml) of lauric arginate addition and 4.6 log CFU/ham in the low level (4 ml) of lauric arginate addition. The authors point out that the surface treatment method requires lesser overall levels of addition compared with internal addition and thus results in a more economical cost of use of antimicrobials (Luchansky et al., 2005). However, all of the exposed surface must be treated and therefore use is mostly restricted to larger, intact meat pieces. Stopforth et al. (2010) also found that lauric arginate as a 0.07% surface treatment gave a 1 log CFU/gram initial reduction of *L. monocytogenes*, but the organism was able to grow at a rate similar to the control, if no additional ingredients were added. If a combination of potassium lactate

and sodium diacetate were added in the formulation and lauric arginate was used as a surface treatment there was no growth throughout a 90-day storage period. Porto-Fee et al. (2010) evaluated the use of 22 or 44 ppm lauric arginate as a surface treatment for frankfurters with and without internal addition of potassium lactate and sodium diacetate. They found that lauric arginate gave an initial reduction of *L. monocytogenes* by 1.5-2.0 log CFU/package immediately after addition, but *L. monocytogenes* was able to grow to ca. 7 log CFU/package over the 120 day shelf life of the frankfurters.

A great number of compounds capable of inhibiting growth of *Listeria* in ready-to-eat meats are available to manufacturers of processed meats now. A listing of ingredients that are approved for use in meat and poultry products is updated by the USDA Food Safety and Inspection Service on a regular basis (FSIS, 2011). This list includes a number of antimicrobial ingredients that can be used in ready-to-eat items to inhibit the growth of *L. monocytogenes*. The list includes cultured sugars, lauric arginate (LAE), nisin preparation, sodium metasilicate, and blends of multiple ingredients intended for use in ready-to-eat products for pathogen control. This list also states the limits in the amounts of these materials that can be added to ready-to-eat items.

### **Species differences**

The previously mentioned study by Glass and Doyle (1989) showed that *L. monocytogenes* grew “exceptionally well” on chicken and turkey products, with an increase of  $10^3$  to  $10^5$  CFU/g within 4 weeks, which was the most rapid growth of all products tested. There was likely an interaction of salt and species, as the sliced turkey formula with a greater concentration of salt (sodium chloride at 2.7%) and carbohydrate had a smaller level of growth compared to the sliced turkey with a reduced concentration

of salt (sodium chloride at 1.3%).

Rapid growth of *L. monocytogenes* in processed poultry products without antimicrobial ingredients has been verified in several studies under either refrigerated or abused storage temperatures. Lianou et al. (2007) evaluated the growth of *L. monocytogenes* in uncured RTE turkey breast commercially available with or without a mixture of potassium lactate and sodium diacetate (therefore, exact formulations were not available), using either immediate inoculation to simulate plant production contamination or following various storage periods of 5-50 days, to simulate home or commercial preparation contamination. Inoculation was 0.1 ml of inoculum to each side of a piece of sliced turkey breast giving a 1-2 log cfu/cm<sup>2</sup> inoculum level. In products without lactate and diacetate, *L. monocytogenes* increased from 1.6 log cfu/cm<sup>2</sup> on day 0 to 7.3 log cfu/cm<sup>2</sup> by day 25. Wederquist et al. (1994) also showed that *L. monocytogenes* grew rapidly in inoculated turkey bologna stored at 4°C. However, Beumer, teGiffel, deBoer, and Rombouts (1996) found very little difference between species as luncheon meat, ham, and chicken inoculated with 10 cfu/g *L. monocytogenes* all grew from the inoculated level to >10<sup>7</sup> cfu/g within 6 weeks when stored at 7°C.

Lin et al. (2006) evaluated the dynamics of transfer of *L. monocytogenes* from a contaminated commercial slicer onto three types of deli meat (bologna, salami, and oven-roasted turkey). The slicer was inoculated at levels of 10<sup>1</sup> and 10<sup>2</sup> CFU before each of the products were sliced and packaged. For the bologna and salami products, *L. monocytogenes* counts decreased during storage period of 90 days and eventually became non-detectable. The greatest number of *L. monocytogenes*-positive samples was detected from the oven-roasted turkey product and the percentage of positive samples increased

for this product throughout the 90 day storage period.

Mujahid et al. (2008) chose to use ready-to-eat sliced turkey meat to measure protein expression due to the findings of other studies (Glass & Doyle, 1989; Farber & Daley, 1994a; Lin et al., 2006; Lianou et al., 2007) that showed that deli turkey meat provides a very favorable environment for growth of *L. monocytogenes*, even during refrigerated storage. Pal, Labuza, and Diez-Gonzalez (2008) showed that strains of *L. monocytogenes* were able to grow at a faster rate in sliced uncured turkey breast than in ham when stored at 4, 8, or 12°C.

Pradhan et al. (2009) conducted a study that evaluated three types of deli meats: ham, turkey, and roast beef. The purpose of the study was to update the original FFRA growth model for listeriosis to estimate risk of listeriosis associated with selected deli meats and model the effect of deli meat reformulation using growth inhibitors on the estimated number of listeriosis cases and deaths. They found that turkey permitted the fastest *L. monocytogenes* estimated growth rate and the shortest lag phase. The authors concluded that reformulation of ready-to-eat deli meats with growth inhibitors is likely to reduce listeriosis cases, but would not be effective in completely eliminating infection caused by *L. monocytogenes*.

### **Temperature differences**

A study by Grau and Vanderlinde (1992) showed that at 0.1°C, *L. monocytogenes* grew slower than other background flora (mostly lactic acid bacteria) in ready-to-eat meat items, but as the storage temperature increased up to 15°C, the rate of growth for *L. monocytogenes* was more rapid than that of the other flora. The authors also found that as the background flora reached counts of approximately  $10^8$  CFU/g, *L. monocytogenes*

growth completely stopped.

Islam, Chen, Doyle, and Chinnan (2002) tested the ability of antimicrobial ingredients to control *L. monocytogenes* growth at 4, 13, and 22°C. They found that sodium benzoate or sodium diacetate solutions sprayed on the meat surface provided inhibition compared to untreated control products, but growth rates tended to be greater at increased storage temperatures.

Pradhan et al. (2010) developed models of *L. monocytogenes* growth which showed that antimicrobial compounds serve as inhibitors to growth of *L. monocytogenes*, but as storage temperatures increase above 5°C, *L. monocytogenes* can grow to levels that can cause illness and mortality. Pal et al. (2008) showed that the combination of potassium lactate and sodium diacetate in ready-to-eat meats was effective in controlling growth of *L. monocytogenes* at 4°C, but did not restrict growth when meats were stored at 8 and 12°C.

### **Irradiation and other potential non-thermal technologies**

An expert panel recommended that the use of processing treatments after packaging was one option to reduce the risk of *L. monocytogenes* growing to large numbers in ready-to-eat products (Walls et al., 2005). One method that has been tested for control of pathogens such as *L. monocytogenes* in ready-to-eat foods is ionizing radiation. This process involves subjecting foods to a level of radiation via gamma rays, X-rays, or machine generated electrons (Rahman, 2007). Fu, Sebranek, and Murano (1995) found that irradiation of cooked pork chops and cured hams at 0.75 to 0.90 kGy reduced counts of *L. monocytogenes* by >2 log cfu/g. Zhu, Mendonca, Ismail, Du, Lee, and Ahn (2005) showed that a 2.0 kGy dose of irradiation decreased *L. monocytogenes*



counts from an initial inoculation level of  $>6.0$  log CFU/g to less than 3.0 log CFU/g after irradiation treatment in turkey ham. Badr (2004) studied use of irradiation to control *L. monocytogenes* in rabbit meat and found that a 3 kGy dose reduced counts of *L. monocytogenes* from an initial count of 3.8 log CFU/g to levels below detection after irradiation treatment. Foong, Gonzalez, and Dickson (2004) evaluated effect of irradiation on *L. monocytogenes* in six different types of ready-to-eat meat items and found that 1.5 kGy irradiation dose gave a 3 log CFU/g reduction, while a 2.5 kGy irradiation dose gave a 5 log CFU/g reduction in *L. monocytogenes*. Zhu, Mendonca, Ismail, and Ahn (2008) showed that a 2.0 kGy dose of electron beam irradiation gave a *L. monocytogenes* reduction of  $>3.0$  log CFU/g in turkey breast and turkey ham products.

Even though irradiation shows the capability of reducing *L. monocytogenes* growth in ready-to-eat products, its use in commercial operations has been limited. Houser, Sebranek, and Lonergan (2003) found that irradiation increased lipid oxidation and caused color changes in hams. Also, consumers have expressed concerns and negative views towards use of irradiation on foods (Manas & Pagan, 2005; Cardello, Schutz, & Leshner, 2007). The FDA has approved irradiation for only single ingredient meat items (Federal Register, 1999) so irradiation cannot be used on most ready-to-eat products which have multiple ingredients. Also the FDA requires use of the radura symbol on labels of food items treated by ionizing radiation and requires a statement on the label of either “Treated with radiation” or “Treated by irradiation” (Code of Federal Regulations, 2010b) which has probably led to some of the negative consumer views of the process (Cardello et al., 2007) and hindered further adoption of this technology.

### **High Hydrostatic Pressure**

A process that has been gaining increased use in commercial ready-to-eat foods is high pressure processing or high hydrostatic pressure. The use of pressure to reduce microbial growth in foods was first investigated late in the 19<sup>th</sup> century by Hite (Hite, 1899; Patterson, 2005), but it wasn't until the 1980's that the technology drew increasing research interest in food processing applications (Rivalain, Roquain, & Demazeau, 2010). The main use of high pressure processing in foods is to extend shelf life and minimize the risk of bacterial pathogens (Martin, Barbosa-Canovas, & Swanson, 2002; Patterson, 2005; Kalchayanand, Sikes, Dunne, & Ray, 1998). The first commercial use of high pressure processing in foods was in high acid jams in Japan in 1992 (Murchie et al., 2005). In the United States, Avomex Inc. (now Fresherized Foods) began using high pressure in 1996 to process avocados and obtain an acceptable shelf life without the use of antimicrobial additives (Torres & Velazquez, 2005). High pressure processing has been used commercially in a variety of other foods including salsa, fruit juices, rice, fish, shellfish, cooked chicken, beef fajita meat, sliced meats, ham, and smoothies (Smelt, 1998; Murchie et al., 2005; Torres & Velazquez, 2005; Campus, 2010). Over 120 processing facilities world-wide are using commercial-scale high pressure processing equipment as of 2008 (Saiz, Mingo, Balda, & Samson, 2008; Campus, 2010), with more than 80% installed after the year 2000 (Balasubramaniam, Farkas, & Turek, 2008).

Several review papers have been written on the effects of high pressure processing on foods, with particular interest on the suppression of bacterial pathogens in ready-to-eat foods (Cheftel, 1995; Smelt, 1998; Farkas & Hoover, 2000; Martin et al., 2002; Patterson, 2005; Balasubramaniam et al., 2008; Aymerich, Picouet, & Monfort, 2008; Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008; Yordanov & Angelova, 2010;

Rendueles, Omer, Alvseike, Alonso-Calleja, Capita, & Prieto 2011). Balasubramaniam et al. (2008) pointed out that high pressure processing is also termed high hydrostatic pressure (HHP) or ultra-high pressure (UHP) in the literature. Smelt (1998) stated that consumer demand for minimally processed and additive-free products have led to further exploration of non-thermal processes such as ultra-high pressure. Balasubramaniam et al. (2008) affirmed that high pressure processing provides the opportunity for processors to manufacture high quality items with fewer ingredients overall and without the use of antimicrobial agents. High pressure processing is an important technology that can help to meet growing consumer demands for natural, minimally-processed, preservative-free products, while maintaining consistent sensory characteristics over an extended shelf life and yet still assure product safety (Considine et al., 2008; Yordanov & Angelova, 2010). Farkas and Hoover (2000) stated that use of high pressure processing for pre-packaged ready-to-eat meats is a viable way to eliminate *Listeria* spp. from products. Rendueles et al. (2011) pointed out that treating pre-packaged sliced meats with high pressure, after the product has already received a full heat treatment, provides a risk minimization for *L. monocytogenes* and other potential contaminants transferred onto the products during slicing and handling. Products treated by high pressure do not require any special labeling in the United States (Aymerich et al., 2008) and the USDA Food Safety and Inspection Service recognizes high pressure processing as an acceptable method for control of *L. monocytogenes* in processed meat products (Balasubramaniam et al., 2008). In fact, the USDA-FSIS issued a letter of no objection to Avure Technologies in 2003 in regard to use of HHP as a post-lethality, post-packaging intervention method to control potential contamination of *L. monocytogenes* in ready-to-eat foods (FSIS, 2004). USDA-

FSIS specifically refers to high pressure processing in subsequent risk assessment documents, stating that high pressure is an example of the type of intervention used in an Alternative 2a (post-lethality intervention) or Alternative 1 process (FSIS, 2007). They further state that high pressure processing lethality is dependent on the time and pressure applied and “is an effective option to eliminate *L. monocytogenes* in the final product and does so without changing the organoleptic qualities of the product” (FSIS, 2007).

Consumer perceptions of high pressure processing of foods have been found to be mostly positive. Cardello et al. (2007) conducted a survey to evaluate consumer perceptions of innovative and emerging food technologies. The technology of high pressure was rated positively by consumers, while technologies of irradiation and genetic modification were rated negatively. Potential product benefits that could be used for marketing of the emerging technologies were also rated by consumers with the benefits of “more nutritious” and “better tasting” drawing positive consumer ratings while, interestingly, “minimally processed” and “fewer preservatives” were rated negatively. Olsen, Grunert, and Sonne (2010) evaluated consumer perceptions of high pressure processing and concluded that consumers were positive to the technology overall, possibly because it was more familiar and easier to understand than other technologies such as pulsed electric fields.

Martin et al. (2002) reviewed high pressure processing of foods and stated that under high hydrostatic pressure, the food is subjected to pressures above 100 MPa with pressures in commercial systems between 400 and 700 MPa. Equipment needed for high pressure processing includes a pressure vessel, a system for providing pressure, temperature control devices, and mechanisms to move products into the system (Martin

et al. 2002; Patterson, 2005). The pressurization is carried out for the duration of the treatment in a confined vessel and in most cases utilizes water as the pressure transmitting medium (Martin et al., 2002). Pressure inside the high pressure vessel is isostatic, or equally applied in all directions, which allows solid foods to retain their original shape (Smelt, 1998; Martin et al., 2002; Patterson, 2005). One of the advantages offered by isostatic compression over thermal treatments is that the process is independent of size and shape of the product being treated (Smelt, 1998; Knorr, Schlueter, & Heinz, 1998; Farkas & Hoover, 2000; Balasubramaniam et al., 2008). Aymerich et al. (2008) reviewed non-thermal pasteurization processes including high hydrostatic pressure and described the process as relatively short in duration, due to the fact that pressure transmission is not size dependent. Martin et al. (2002) also reported that because high hydrostatic pressure does not use heat, the key sensory and nutritional properties of the product are not significantly changed during high pressure processing. High hydrostatic pressure does not affect covalent bonds and therefore has minimal effects on flavor, vitamins, and pigments and is a more gentle process in that regard than other processing methods (Cheftel & Culioli, 1997; Farkas & Hoover, 2000; Patterson, 2005; Yordanov & Angelova, 2010).

One disadvantage of high pressure processing is that it is a batch process, as the vessel needs to be loaded with the food material, the pressure is applied, then pressure is released, and the food product is unloaded before the next batch can be processed (Farkas & Hoover, 2000). The overall costs of high pressure processing are normally greater than addition of antimicrobial ingredients due to the reduced processing throughput (batch process) and a greater initial capital investment (Farkas & Hoover, 2000; Aymerich et al.,

2008). The overall equipment cost for commercial-scale high pressure processing equipment is in the range of \$500,000 to \$2,500,000 per unit (Balasubramaniam et al., 2008; Campus, 2010). Operating process cost has been estimated at 14 eurocent/kg of product (which equates to \$0.088/lb. as of March 1, 2011) treated at 600 MPa, including investment and operation costs (Anon., 2002 as quoted by Aymerich et al., 2008) and at 0.10 - 0.50 eurocent/kg (or \$0.07 to \$0.32 per lb. as of March 1, 2011) (Cheftel & Cuiloi, 1997). Saiz et al. (2008) gave an estimated processing cost for high pressure of \$0.04 to \$0.10 per pound. Farkas and Hoover (2000) stated that high pressure process hold times need to be ten minutes or less at pressure to make the process economically feasible for food processors.

#### Pressure Level

Pressure is defined as “the force per unit area applied on a surface in a direction perpendicular to the surface” (Rivalain et al. 2010). The official pressure unit is Pascal (Pa), but since this is a very small level of pressure the megapascal (MPa) is used as the common pressure unit described in most high pressure studies (Rivalain et al., 2010). One atmosphere is equal to 9.9 MPa and one MPa is equal to 145 pounds per square inch (psi) (Rivalain et al., 2010). Pressurization of a high pressure vessel can be accomplished either by direct compression utilizing a piston to pressurize the fluid or, in most cases, indirect compression via a high-pressure intensifier to transfer the fluid medium from a holding tank into the high pressure vessel to achieve the desired pressure (Yordanov & Angelova, 2010).

Because the pressure is applied isostatically in the pressure vessel, inactivation of vegetative cells is fairly uniform within the pressure vessel (Smelt, 1998; Campus, 2010).

Farkas and Hoover (2000) pointed out that increasing the level of pressure, time under pressure, or processing temperature will increase the number of microorganisms killed or injured, although there is a minimum pressure required for any inactivation to occur.

Garriga, Aymerich, Costa, Monfort, and Hugas (2002) stated that naturally contaminated meats contain levels of pathogenic and spoilage organisms at a very low levels and that treatment by 600 MPa of pressure is a valid process to inhibit or delay growth of spoilage microorganisms. Yordanov and Angelova (2010) also reported that high pressure processing is carried out for most applications, at about 600 MPa pressure for three to five minutes. Aymerich et al. (2008) reported high pressure treatment is generally in the range of 300–600 MPa for a short period of time, giving inactivation of >4 log units of the vegetative pathogenic and spoilage microorganisms.

In a review of high pressure as a new technology in meat processing, Hugas, Garriga, and Monfort (2002) stated that the effect of come-up time, depressurization time, and process temperature require further investigation. A slow ramp-up time may cause a stress response and make the process less effective, whereas a faster depressurization rate may improve inactivation. They also concluded that high pressure did not change proximate composition of the cooked ham, dry cured ham, or marinated beef loin, and that products vacuum packed and high pressure treated at 600 MPa for 10 minutes at 30°C are not different in flavor compared to the same untreated products (Hugas et al., 2002).

Youart, Huang, Stewart, Kalinowski, & Legan (2010) showed that *L. monocytogenes* inoculated into uncured chicken and turkey samples with 1.2-1.3% salt were completely inactivated when high pressure treated at 600 MPa for 210 seconds.

Youart et al. (2010) also found that time under pressure had a significant effect on inactivation of *L. monocytogenes* in pressure-treated samples, with increased time under pressure giving increased inactivation of *L. monocytogenes*.

Jofre, Aymerich, Grebol, and Garriga (2009) reported that the application of a high pressure treatment of 600 MPa for 6 minutes at 31°C was shown to be highly effective for decreasing bacterial load and extending the shelf-life of refrigerated ready-to-eat meat products while improving their safety. Their testing at these parameters showed that *Salmonella* and *L. monocytogenes* were effectively inactivated with counts reduced from approximately 3.5 log CFU/g to <10 CFU/g in all the products. The same study showed that among spoilage organisms, lactic acid bacteria counts were reduced by 4.6 log CFU/g in cooked ham.

An earlier study by Aymerich et al. (2005) used an inoculation of *L. monocytogenes* at  $10^2$ - $10^4$  CFU/g in sliced cooked ham. The results showed that high hydrostatic pressure treatment (400 MPa/10 min. at 17°C) reduced the number of viable cells of *L. monocytogenes* and growth was inhibited in all treatments until 42 days of storage when stored at 1°C or 6°C. After this time some products stored at 6°C experienced growth of 2-4 log CFU/g by the end of the 84 day storage period.

In a study from Spain, Marcos, Aymerich, Monfort, and Garriga (2008) tested high pressure processing to control growth of *L. monocytogenes* during product storage. The product test was termed cooked “ham” (but used raw materials from pork shoulder) and contained about 1.80% sodium chloride and 100 ppm of sodium nitrite. After cooking, the product was inoculated with  $10^4$  CFU/g of a 3-strain mixed culture of *L. monocytogenes*. High pressure processing consisted of pressurization of 400 MPa for 10



minutes at 17°C. Control product showed rapid growth of *L. monocytogenes*, reaching >8 log CFU/g in 22 days. High pressure processing produced an immediate reduction of *L. monocytogenes* population of 3.4 log units, but when stored at 6°C, samples grew back to 4 log CFU/g within 22 days and were not different than control product after 30 days. High pressure treatment using 400 MPa for 10 minutes at 17°C followed by storage at 1°C reduced *L. monocytogenes* to around the detection limit and maintained this level throughout the 60 day storage period (Marcos et al., 2008).

Pietrzak, Fonberg-Broczek, Mucka, and Windyga (2007) studied the effect of high pressure treatment on the quality of cooked, sliced pork ham at 600 MPa for 10 minutes at 20°C. The authors reported that high pressure treatment resulted in a significant improvement to product shelf life but did not significantly affect texture. Drip loss was significantly increased, showing that high pressure may have some negative effects on water holding and purge loss in ready-to-eat products (Pietrzak et al. 2007).

Overall, a significant increase in shelf life can be achieved by use of high pressure, however the process does not provide sterilization of the products, so refrigerated storage throughout the shelf life of the item is required (Considine et al., 2008; Rendueles et al., 2011).

#### Mode of effect

The mechanisms for inactivation of bacteria by high pressure treatment are not as clearcut as those for some other non-thermal technologies (Manas & Pagan, 2005).

Farkas and Hoover (2000) grouped the effects of high hydrostatic pressure on reduction and inactivation of vegetative organisms into four groups: 1) cell wall and membrane effects, 2) pressure-induced cellular changes, 3) biochemical changes, and 4) genetic

mechanism effects. Others have researched the effects of these four modes of inactivation or their combination and presented various conclusions and theories.

Several researchers have pointed to the breakdown of the cell membrane as the key mechanism for bacterial reduction caused by high pressure (Cheftel, 1995; Smelt, 1998; Ritz, Tholozan, Federighi, & Pilet, 2001; Patterson, 2005). Ritz et al. (2001) theorized that destruction of cell walls or membranes could be the main mechanism for microbial reduction. They cautioned though that their evaluation of injury of *L. monocytogenes* in citrate buffer at 400 MPa showed that a small minority of cells had only slight damage to the cell membrane.

Rivalain et al. (2010) reported that cell membranes are thought to be the location for initial injury of bacterial cells during high pressure treatment. Ritz, Tholozan, Federighi, and Pilet (2002) used scanning electron microscopy to show some areas of injury on the surface of *L. monocytogenes* cells subjected to pressure treatment. They showed that cell wall or membrane injury is a likely mechanism for bacterial injury and/or death via high pressure treatment. Ritz et al. (2002) pointed out that the degree of microbial injury is not always homogeneous and it is possible that damage to some cells could be repaired over time so that they could grow and reproduce. Cell membrane pressure damage may also cause trigger further potential changes in cell permeability, nutrient transport, loss of osmotic balance, and loss of pH regulation (Aymerich et al. 2008) and therefore it is difficult to understand the exact mechanism of microbial inactivation.

Kalchayanand, Dunne, Sikes, and Ray (2004) showed that *L. monocytogenes* cells had only a slight irregular surface after pressurization, even with a 1 log kill, but after two

hours of incubation, the cell surface appeared rough and quite different from normal cells. Smelt (1998) pointed out that inactivation of cells via high pressure is accompanied by an increase in the amount of ATP on the cell surface, which was apparently leached from the cell cytosol and was a further indication that membrane damage had occurred. Farkas and Hoover (2000) pointed out that leakage of components from microbial cells was an indicator that some level of membrane damage had occurred and greater loss of intracellular material was correlated with greater levels of cell injury and death.

However, some researchers have noted that extensive cell membrane damage does not occur in some organisms until after cell death (Ritz et al., 2001; Ritz et al., 2002; Ananta & Knorr, 2009). Pagan and Mackey (2000) reported that *E. coli* cells subjected to high pressure can maintain an intact cytoplasmic membrane even in dead cells. So other mechanisms may be involved in inactivation of bacterial cells by high pressure (Rivalain, et al., 2010), including protein coagulation, and key enzyme inactivation (Manas & Pagan, 2005). Klotz, Manas, and Mackey (2010) observed that at pressures above 400 MPa, the amount of protein that leaked through the cell membrane was not as great as at lesser pressure levels, leading the authors to conclude that some aggregation of protein in the cell had occurred and resulted in larger proteins that were not able to pass through the cell's peptidoglycan layer.

Hayman, Kouassi, Anantheswaran, Floros, and Knabel (2008) showed that when cells were suspended in solutions with a water activity below 0.83 the effectiveness of high pressure was significantly reduced. Effects of high pressure processing on lactate dehydrogenase were evaluated. Samples that were untreated showed no aggregation,

indicating that the enzyme remained stable, but when the enzyme was exposed to 600 MPa, enzyme aggregation occurred, showing that the stability of the enzyme was reduced. The authors concluded that since high pressure processing induced misfolding and aggregation of lactate dehydrogenase, it is probable that high pressure has similar effects on other proteins, including proteins within *L. monocytogenes*. Therefore the effect of high pressure in causing misfolding of any microbial cellular proteins including enzymes or membrane proteins could make these proteins incapable of performing their normal functions within the cells (Hayman et al., 2008) and thus greatly decreasing the capability of the cell to repair and reproduce.

#### High Pressure Resistance and Barotolerance

When microorganisms are subjected to heat, their death rate gives a fairly linear slope, but high pressure inactivation does not always follow first-order kinetics (Klotz, Pyle, & Mackey, 2007). Some researchers have found death rates closely follow first-order kinetics (Mussa, Ramaswamy, & Smith, 1999; Ponce, Pla, Mor-Mur, Gervilla, & Guamis, 1998), while others have found a non-linear relationship with a tailing behavior in seemingly pressure-tolerant survivors (Farkas & Hoover, 2000; Smelt, Hellemons, Wouters, & van Gerwen, 2002). Klotz et al. (2007) developed a new mathematical modeling approach for predicting high pressure inactivation in which the model assumes that inactivation by pressure is a first-order inactivation by which the rate of inactivation increases with the reciprocal of the square root of time. This model gave a good fit to the wealth of scientifically-published data available for high pressure inactivation (Klotz et al., 2007).

Tay, Shellhammer, Yousef, and Chism (2003) tested death kinetics of nine strains

of *L. monocytogenes* and one strain of *L. innocua* in tryptose broth. All strains showed non-first-order kinetics with a quick initial drop in numbers, followed by a diminished rate of death. When strains were evaluated at 800 MPa, an 8-log reduction was observed during the come-up time (~3 min.) with minimal further reduction during an eight-minute hold time at pressure.

Gram-positive bacteria are more barotolerant than Gram-negative bacteria, probably as a result of cell membrane differences as Gram-positive bacteria have a greater percentage of peptidoglycan and teichoic acids than Gram-negative bacteria (Russell, Evans, terSteeg, Hellemons, Verheul, & Abee, 1995; Smelt, 1998; Lopez-Caballero, Carballo, & Jimenez-Colmenero, 1999; Patterson, 2005). Farkas and Hoover (2000) reported that the degree of pressure resistance is related to the cell's ability to repair membrane damage caused by high pressure treatment. They further reported that cells in stationary growth phase are able to repair membrane damage more easily than cells that are in the exponential growth phase. Metrick, Hoover, and Farkas (1989) reported a tailing effect of different *Salmonella* strains subjected to high pressure treatment and further reported that when surviving strains were re-introduced into product, they were found to be not significantly different in barotolerance than the original strain.

Simpson and Gilmour (1997) tested the effect of high pressure on three strains of *L. monocytogenes* (NCTC 11994, an unidentified strain, and Scott A) in phosphate-buffered saline and found a tailing effect, especially at the high pressure levels tested (375-450 MPa). The authors concluded that *L. monocytogenes* barotolerance is dependent on the specific strain being tested.

Jofre, Aymerich, Bover-Cid, and Garriga (2010) tested 5 strains of *L. monocytogenes* in broth and found that after 400 MPa pressure for 10 minutes, all strains were below detection limits (<100 CFU/ml), but all strains showed recovery when stored at 14°C or 22°C. After 10 minutes of high pressure treatment at 600 MPa, four of the five strains showed similar recovery, but at lesser levels. After 5 minutes of high pressure treatment at 900 MPa, only one of the five strains showed any recovery over the 21 day test.

Hayman, Baxter, O'Riordan, and Stewart (2004) showed that the pressure resistance of *L. monocytogenes* was increased as the salt concentration of the product was increased. In a study of *L. monocytogenes* growth in milk, Shearer, Neetoo, and Chen (2010) found that a reduced level of inactivation by high pressure was achieved when *L. monocytogenes* was grown at 40-43°C (0.5-1.5 log) compared with *L. monocytogenes* grown at 4-35°C (>4.5 log reduction). This shows that there could be some level of barotolerance in milk samples depending on time of *L. monocytogenes* infection and handling conditions. If milk is added as an unpackaged fluid to high pressure unit (as the pressurizing medium), then a small amount of milk will remain after each batch and give the potential for *L. monocytogenes* cells to remain and be exposed to more than one pressure cycle, allowing a potential for adaptation to the high pressure conditions. This type of adaptation or barotolerance is less likely in ready-to-eat meats that are fully packaged after already undergoing a lethality heat process.

Kato and Hayashi (1999) also reported that high pressure induces the phase transition of natural membranes, which leads to a decrease in membrane fluidity, and this may result in breaking of the membrane. Although phase transition of membrane lipids

is not necessarily lethal to bacteria, it has been demonstrated that increased membrane fluidity is linked to increased pressure resistance (Casadei, Manas, Niven, Needs, & Mackey, 2002). Similarly, Braganza and Worcestor (1986) indicated that exposure to pressure increases the packing density of lipids in membranes and promotes phase separation due to compressibility differences of lipids and proteins in the cell membrane. Therefore, differences in membrane composition may be one of the reasons there are different pressure sensitivities among various strains of *L. monocytogenes* (Braganza & Worcestor, 1986).

Oceans have an average pressure of about 38 MPa and a maximum pressure of about 110 MPa (Bartlett, 2002), so organisms living in underwater environments provide some indication of how bacteria could withstand exposure to high levels of pressure. Kato and Hayashi (1999) theorized that an increase in the ratio of unsaturated fatty acids to saturated fatty acids in membranes of deep-sea bacteria may explain much of their barotolerance. They also suggest that polyunsaturated fatty acids are responsible for maintaining membrane fluidity in these organisms during their exposure to high pressure conditions. Winter and Jeworrek (2009) stated that membranes can alter their degree of saturation, and thus their melting point, as an adaptation to temperature or pressure changes. So it is therefore possible that organisms can alter their membrane lipid configuration to increase their tolerance to pressure. Bartlett (2002) pointed out that membranes are extremely susceptible to changes in pressure, but as modeled by some deep sea organisms, they do have capability to adapt to pressure changes. One theory of why membranes adapt to pressure is so that they can maintain permeability to allow certain materials such as ions to flow through the membrane (Bartlett, 2002; Van De

Vossenbergh, Ubbink-Kok, Elferink, Driessen, & Konings, 1995). This potential change in fluidity may allow addition of certain materials in conjunction with high pressure that will serve to weaken the membrane and potentially provide additive or synergistic effects for microbial pathogen control in food items when exposed to high hydrostatic pressure.

Jofre et al. (2009) reported that low water activity decreases the efficiency of high pressure treatment. In their evaluation of high pressure treatments on several meat items, dry cured ham recorded the lowest levels of high pressure kill, but no further growth was observed during subsequent storage for 120 days at 4°C.

#### Temperature effect

The high pressure process is a non-thermal treatment, although adiabatic heating does add about 2-3°C for each 100 MPa (Aymerich et al., 2008; Cheftel & Culioli, 1997). Yordanov and Angelova (2010) also reported that during pressurization, the temperature of the food in the high pressure vessel increases as a result of the physical compression, with this compression causing a reduction in volume of approximately 15% at 600 MPa and therefore a reduction of similar size for foods with high moisture and low gas levels (Cheftel & Culioli, 1997; Balasubramaniam et al., 2008). The product will return to a temperature very close to the initial temperature after depressurization when a short pressurization process is performed (Cheftel & Culioli, 1997; Farkas & Hoover, 2000; Yordanov & Angelova, 2010).

Chen (2007) conducted high pressure treatment at various temperatures and determined that the pressure resistance of *L. monocytogenes* was more dependent on treatment temperature if the temperature was outside of the range of 10-30°C. *L. monocytogenes* was somewhat more sensitive to pressure treatment at 1°C than at 10-



30°C and was most resistant to pressure at temperatures between 10 and 30°C. At above 30°C, there were pressure and temperature interactions that increased the *L. monocytogenes* kill. At 500 MPa (for 1 min.) samples at 0 – 30°C gave ~1.0-1.6 log decrease, whereas 40°C gave a 4 log decrease, and 50°C gave a >6 log decrease in *L. monocytogenes*. Adiabatic heating resulted in temperature increases in product of 15-20°C at greater processing temperatures (40°C and 50°C) and contributed to the *L. monocytogenes* log reduction (Chen, 2007). Simpson and Gilmour (1997) also found that there was an increase in *L. monocytogenes* kill with high pressure at 45°C compared to products treated with the same level of pressure at ambient (18°C) temperature.

Chen (2007) also found that as treatment time increased, the death rate of *L. monocytogenes* increased. For each pressure level, the log reduction curves show a rapid initial drop in counts followed by tailing caused by a decreased inactivation rate, indicating a minor portion of organisms may be more pressure resistant. Simpson and Gilmour (1997) found that a high pressure of 375 MPa at 45°C for 5 minutes in cooked minced beef gave a 4.5 log reduction in *L. monocytogenes*, but extending the time up to 30 minutes resulted in almost no additional inactivation of *L. monocytogenes*. Chen (2007) found that a non-linear (Weibull) distribution model gave a better representation of observed inactivation than a linear model. The Weibull distribution is a statistical model that can be used to fit non-linear data from a variety of fields (Rinne, 2009). Buzrul, Alpas, and Bozoglu (2005) also found a tailing effect due to high pressure treatment and found that the Weibull model provided a good fit to the data. Buzrul and Alpas (2004) studied survival rate of *Listeria innocua* subjected to pressures of 138-345 MPa and temperatures of 25-50°C. They found a significant tailing effect and in

evaluation of Weibull and log-logistic models, the log-logistic model had a better fit, although both were better than a linear model. The log-logistic model provides good fit for data with a long tailing effect, so is appropriate for the high pressure data presented by Buzrul and Alpas (2004).

Escrui and Mor-Mur (2009) tested the effect of high pressure on general and specific media to study possible interactions of fat and high pressure on the growth of *Listeria innocua* and *Salmonella* Typhimurium. The study showed that Gram-negative bacteria (*S. Typhimurium*) were more susceptible to high pressure than Gram-positive bacteria (*L. innocua*). *Listeria innocua* reduction during high pressure was influenced by the type of fat (olive>linseed> tallow), but not by the level of fat.

Overall, the tailing effect observed in numerous studies shows that high pressure inactivation of bacteria, and specifically *L. monocytogenes*, does not follow first-order kinetics. This also means that sub-lethal injury can occur in some cells subjected to high pressure and those organisms may recover and grow, if given a sufficient period of time at a temperature that is adequate for growth (Bozoglu, Alpas, & Kaletunc, 2004). Any studies of products should include a design to test the ability of these organisms to recover and grow during the product shelf life (Patterson, Quinn, Simpson, & Gilmour, 1995; Ritz, Pilet, Jugiau, Rama, & Federighi, 2006; Ulmer, Ganzle, & Vogel, 2000; Rendueles et al., 2011). Also, there seems to be a somewhat protective effect on *L. monocytogenes* when subjected to high pressure treatment in food compared to treatment in a broth, buffer, or media solution (Patterson, et al. 1995; Smelt, 1998; Campus, 2010). Therefore it is important to test the effect of high pressure in each product and formulation that will actually be made, rather than testing in a buffer solution, so that the

true effect of high pressure treatment of a specific food product can be determined (Campus, 2010).

### **Combinations of high hydrostatic pressure and ingredient addition**

Aymerich et al. (2005) showed product that was treated by high pressure, but with no antimicrobial additives, had reduced *L. monocytogenes* levels immediately after high pressure (400 MPa, 17°C, 10 min.) compared to a second product not treated with high pressure, but with lactate added to the formulation (1.8% potassium lactate solids). However, after 84 days of storage, the high pressure product had similar levels of *L. monocytogenes* as the non-high pressure product with lactate, when stored at 6°C. Products treated at the same pressure level but with added nisin, showed some growth by day 84 of storage at 6°C, but at a reduced level compared to the high-pressure control product. The addition of lactate to high-pressure treated product completely inhibited growth during storage at 6°C.

Jofre et al. (2007) studied the ability of interleaved paper containing bacteriocins and/or potassium lactate (1.8%) to inhibit *L. monocytogenes* in conjunction with high pressure processing (HPP) at 400 MPa. The interleavers were placed between slices of ham that were inoculated at  $3 \times 10^4$  CFU/g and packaged before high pressure treatment at 400 MPa for 10 minutes. Products treated with bacteriocins showed a reduction of 4.2 to 4.5 logs after high pressure processing, whereas control and lactate-only products had a log-reduction of only 1.76 and 1.5 log CFU/g, respectively. Interleavers containing a combination of lactate and nisin were best at inhibiting growth and did so for 30 days. At the end of storage (90 days), this treatment had significantly less growth than the control (1.88 log units less). In non-high pressure-treated packages, the combination of nisin and

lactate was most effective. In comparing the results of this study which used nisin on interleavers with a previous study using nisin in the formulation (Aymerich et al., 2005), the authors concluded that the increased efficacy of nisin when administered through interleavers is likely due to its application on the surface of the slices, where the contamination is also occurring.

Chung et al. (2005) reported that there was no clear relationship between the sensitivity to nisin and high pressure shown by Tay (2003) among different *L. monocytogenes* strains. Chung et al. (2005) concluded that high pressure may target cell membranes, but the mechanism of membrane damage caused by high pressure is probably different from the damage caused by nisin. When evaluated in phosphate buffer, effects of nisin plus high pressure apparently acted additively, whereas when cells were pretreated with nisin and TBHQ, then high pressure processed, the combined factors acted synergistically and inactivated 7.3 logs. When tested in sausage items, addition of TBHQ, nisin, or a combination greatly enhanced the effect of high pressure processing when compared to high pressure used alone (10-30% of samples positive for *L. monocytogenes* when TBHQ and/or nisin were added, vs. 85-95% positive with high pressure alone).

Chung and Yousef (2008) showed that the combination treatment of TBHQ (50 ppm), dissolved in dimethyl sulfoxide (DMSO), and high pressure processing resulted in synergistic inactivation of *L. monocytogenes* strains at 400 and 500 MPa. The authors concluded that DMSO was needed as a solvent to assist the highly hydrophobic TBHQ in destruction of pathogens in an aqueous environment. The most probable action site of DMSO on vegetative organisms is thought to be by breaking down cell membranes (Yu

& Quinn, 1998).

Kalchayanand et al. (2004) showed that nisin is effective against growing cells, while pediocin is effective against both growing and non-growing cells of gram-positive bacteria. Scanning electron micrographs showed cell membrane breakdown in bacteriocin-treated cells and a greater death rate of *L. monocytogenes* in treatments with bacteriocins compared to those treated with high pressure alone. Pressurization (345 MPa for 5 minutes at 25°C) in treatments with added bacteriocin showed partial collapse of *L. monocytogenes* cells immediately after high pressure treatment with increased levels of cell breakdown after 120 minutes. The authors concluded that *L. monocytogenes* inactivation following treatment with pediocin and nisin or high pressure is caused by cell wall and cell membrane damage (Kalchayanand et al., 2004).

Marcos et al. (2008) studied the application of high pressure processing, bacteriocin packaging film, and the combination of the two in controlling growth of *L. monocytogenes* during product storage. Cooked ham (from pork shoulder) was used for the meat and contained about 1.80% sodium chloride and 100 ppm of sodium nitrite. Samples were high pressure processed for 10 minutes at 400 MPa. When a bacteriocin-containing film was added between slices of ham and used in combination with high pressure, both the control high pressure process and the high pressure with bacteriocin film gave an immediate *L. monocytogenes* reduction of about 1.5 log CFU/g. However after storage, the product containing the bacteriocin film maintained a *L. monocytogenes* level of < 2.0 logs throughout the 60 day storage period, while the control high pressure product grew sturdily to ~8 log CFU/g by the end of the 60 day storage period.

## **Growth of Natural Foods**

There has been a dramatic growth of foods that are labeled natural, minimally-processed, or preservative-free over the past several years. It is estimated that sales of natural foods in the United States will reach \$30 billion by 2014 (Nunes, 2011). One area of this growth has been in ready-to-eat meat products labeled as “Natural” or “Preservative-free.” These products must meet additional labeling requirements as detailed in the Code of Federal Regulations (2010a), 9 CFR 317.17 and 9 CFR 319.2, including having the term “Uncured” before the product name if it is “found by the Administrator to be similar in size, flavor, consistency and general appearance to such products as commonly prepared with nitrate and nitrite.” Sebranek and Bacus (2007) stated that these natural or preservative-free products must be free of chemical preservatives, but in many cases use vegetable-based ingredients with high natural nitrate content and starter cultures to produce “naturally cured” items with similar characteristics to traditional cured meat items. In an evaluation of commercial ham, bacon and frankfurters, Sindelar, Cordray, Olson, Sebranek, and Love (2007) showed that overall consumer liking for one brand of natural products (uncured, no-nitrate/nitrite added) was equal to that of a standard product (sodium nitrite-added). Sindelar, Cordray, Sebranek, Love, and Ahn (2007) found that uncured, no-nitrite added products at a low concentration of vegetable juice powder (0.20%) had similar sensory scores to products conventionally-cured using sodium nitrite, however, the residual nitrite levels of these items were much less than the conventionally-cured products and thereby had a potential for increased food safety risk. Sebranek and Bacus (2007) pointed out that the food safety of naturally cured items is an area that requires more research to adequately assess.

Jackson, Sullivan, Kulchaiyawat, Sebranek, and Dickson (2011) showed that naturally-cured products have a greater level of *Clostridium perfringens* growth than conventionally cured products, leading the authors to conclude that additional food safety measures should be used to assure an adequate level of safety in these products, which do not have direct addition of sodium nitrite.

### **Overall Costs of Food Spoilage**

In addition to illnesses caused by foodborne pathogens, there is a large amount of food that is wasted each year, even though there is a great need for food in some parts of the world. The Food and Agriculture Organization (FAO) of the United Nations estimates that 925 million people were undernourished in 2010 (FAO, 2010). The United States Government Accountability Office (GAO) estimates that food losses for 2008 for meat, fish, eggs, and nuts from retail establishments at 5.2% (12.5 lbs./capita/yr.) and losses from consumer use are another 31.7% of retail weight (75.8 lbs./capita/yr.), based on a per capita consumption, adjusted for loss, of 150.9 pounds per year (GAO, 2011). The 2008 loss data for total red meat is 4.9 lbs./capita/year loss at retail and 36.2 lbs./capita/year loss at consumer use and for total poultry is 7.1 lbs./capita/year loss at retail and 26.8 lbs./capita/year loss at consumer use, based on per capita consumption, adjusted for loss, of 67.2 pounds per year of red meat and 43.0 pounds per year of poultry (GAO, 2011). While some of these losses are due to moisture/fat loss during cooking and waste from parts such as bones that are not edible, this still represents a large amount of food that is wasted. The current world population is estimated at about 6.9 billion people as of February, 2011 (Census.gov., 2011c) and FAO

(2011b) has estimated that the world population will grow to around nine billion people by 2050 and by that time global demand for food will nearly double. Use of ingredients and processes that can also minimize food spoilage and increase product shelf-life would be beneficial in helping to feed the growing world population.

### **Overall Hypothesis and Objectives for Proposed Studies**

One research area that has not been addressed in scientific literature is the effect of meat or poultry species on high pressure processing of ready-to-eat meat products. Preliminary research (Myers, 2010 - unpublished data) has shown that *L. monocytogenes* inactivation due to high pressure processing is less in fully-cooked, ready-to-eat turkey products than in similar ham or roast beef items. However, since other differences exist in moisture, fat, protein, salt level, nitrite inclusion, sweeteners used, and other formulation differences, it is unknown if the difference in *L. monocytogenes* inactivation is due to a species effect or some other combination of variables. Several researchers have identified ready-to-eat turkey as an excellent growth media for *L. monocytogenes* (Glass & Doyle, 1989; Schlyter et al., 1993; Wederquist et al., 1994; Burnett et al., 2005; Lin et al., 2006; Lianou et al., 2007; Pradhan et al., 2009; Pradhan et al., 2010), but no testing of species effect due to high pressure processing has been conducted. Therefore, the first phase of this research project will be to determine if differences exist between turkey and ham (pork) with respect to *L. monocytogenes* inactivation in ready-to-eat products due to high pressure processing. Raw materials of pork ham and turkey breast will be procured at a fat level that is very similar (1.0-1.5% fat target). Because pork ham and turkey breast meats have different levels of protein, even at the same fat level



(Myers, 2010 - unpublished data), the water amounts in formulations will be adjusted slightly to minimize differences in finished product protein and moisture. Formulations of both meat sources will be developed using the exact same dry ingredient levels to eliminate these formulation differences.

Another area of ready-to-eat products that is gaining increased consumer spending (Nunes, 2011), and increased scientific research (Sebranek and Bacus, 2007; Sindelar, Cordray, Olson et al., 2007, Sindelar, Cordray, Sebranek, et al., 2007; Jackson et al., 2011), is the area of natural food products. Sodium nitrite has been identified by several researchers as an inhibitor of *L. monocytogenes* growth in ready-to-eat meat products (Grau & Vanderlinde, 1992; Duffy et al., 1994; Farber & Daley, 1994b; Vitas et al., 2004), but no testing of the effect of natural curing ingredients (vegetable juice powder or other ingredients with high levels of nitrate) and interaction with high pressure processing has been conducted. Therefore, the second section of research will focus on the inhibition of *L. monocytogenes* by sodium nitrite in “conventional” USDA standard of identity ham formulations compared to nitrate that has been converted to nitrite in commercially-available fermented vegetable juice powder that provides the nitrite source for naturally-cured meat products.

The third area of research will focus on the combined use of high pressure and antimicrobial treatment to inactivate *L. monocytogenes* at reduced levels of pressure using commercially viable pressure hold times ( $\leq 6$  minutes). Antimicrobial ingredients or processing aids which meet the definition for natural products will be tested, as will those antimicrobials that can be used in conventional products.

All challenge tests will be conducted using a five strain mixed culture of *L.*

*monocytogenes* with each strain acquired as the result of its contamination of foods.

Product manufacture, challenge study tests, and high pressure of products will all take place at the Hormel Foods Research and Development Center in Austin, Minnesota.

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## **CHAPTER 2 - Growth of *Listeria monocytogenes* on RTE ham and turkey with and without use of high hydrostatic pressure**

A paper to be submitted to the journal *Meat Science*

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### **Abstract**

Growth of *L. monocytogenes* was evaluated for up to 182 days after inoculation in ready-to-eat (RTE) sliced ham and turkey breast formulated with sodium nitrite (0 or 200 ppm), sodium chloride (1.8% or 2.4%), and treated (no treatment or 600 MPa) with high hydrostatic pressure (HHP). HHP at 600 MPa for three minutes resulted in a 3.85-4.35 log CFU/g reduction in *L. monocytogenes*. With formulations at similar proximate analyses, one evaluation day without HHP (day 21) showed significantly greater growth of *L. monocytogenes* in ham than in turkey breast, but there were no significant differences on other evaluation days or with HHP. There were no differences in growth of *L. monocytogenes* due to sodium chloride level. Sodium nitrite provided a small, but significant inhibition of *L. monocytogenes* without HHP, but addition of sodium nitrite did not significantly affect growth of *L. monocytogenes* with use of HHP.

### **1. Introduction**

*Listeria monocytogenes* is pervasive in many environments and has the unique ability among pathogens to grow at refrigerated temperatures, making it a pathogen of upmost concern for ready-to-eat (RTE) foods consumed without recooking (Faber & Peterkin, 1991; Kathariou, 2002). *L. monocytogenes* contamination for RTE meat items



occurs mostly during post-lethality processes such as slicing, packaging, or other handling of product (Lin et al., 2006; Vorst, Todd, & Ryser, 2006; Nesbakken, Kapperrud, & Caugant, 1996). An outbreak of listeriosis in 1998 that caused approximately 108 illnesses and 18 deaths was traced to post-lethality contamination at a commercial processing facility (CDC, 1999; Graves et al., 2005).

Oven Roasted Turkey is a commercial product that has been shown to have greater likelihood of *L. monocytogenes* growth compared to other types of meats, such as bologna and salami (Lin et al., 2006). If turkey is contaminated with *L. monocytogenes*, it has the potential to grow to a high level in a short period of time (Lianou, Geornaras, Kendall, Scanga, & Sofos, 2007b; Ojeniyi & Bisgaard, 2000). In the risk assessment published by Pradhan, Ivanek, Grohn, Geornaras, Sofos, & Wiedmann (2009), *L. monocytogenes* had the greatest estimated growth rate and the shortest lag phase in turkey, compared to other products modeled.

Ham items have also been shown to support *L. monocytogenes* growth (Glass & Doyle, 1989; Lianou et al., 2007a). However, in the risk assessment conducted by Pradhan et al. (2009), ham was found to have a reduced growth rate and an extended lag phase of *L. monocytogenes* compared to turkey or a generic deli meat product.

A panel of microbiologists, food scientists, and physicians from government, academia, and industry recommended that the use of post-packaging intervention treatments to reduce the risk of *L. monocytogenes* growing to large numbers in ready-to-eat products (Walls et al., 2005). High hydrostatic pressure (HHP) has been used for inactivation of pathogens in food items, with damage to the cell membrane being the primary mechanism of microbial destruction (Hugas, Garriga, & Monfort, 2002). HHP

can also reduce the levels of vegetative bacteria without greatly affecting the flavor of foods (Cheftel & Culioli, 1997). High hydrostatic pressure treatment of meats after packaging has been shown to reduce the number of *L. monocytogenes* in hams (Aymerich, Jofre, Garriga, & Hugas, 2005; Pietrzak, Fonberg-Broczek, Mucka, & Windyga, 2007; Marcos, Aymerich, Monfort, & Garriga, 2008; Jofre, Aymerich, Grebol, & Garriga, 2009), cooked beef (Simpson & Gilmour, 1997b), and cooked poultry products (Youart, Huang, Stewart, Kalinowski, & Legan, 2010). Researchers have also shown that sodium nitrite (Buchanan, Stahl, & Whiting 1989; Buchanan & Phillips 1990; McClure, Kelly, & Roberts, 1991; Schlyter, Glass, Loeffelholz, Degnan, & Luchansky, 1993; Grau & Vanderlinde 1992; Duffy, Vanderlinde, & Grau, 1994; Farber & Daley, 1994b; Vitas, Aguado, & Garcia-Jalon, 2004) and sodium chloride (Glass & Doyle 1989; McClure et al. 1991; Seman, Borger, Meyer, Hall, & Milkowski, 2002; Legan, Seman, Milkowski, Hirschey, & Vandeven, 2004) can reduce the growth rate of *Listeria monocytogenes*.

The purpose of this study was to determine the extent to which species, salt concentration, sodium nitrite concentration, and HHP influence the growth of *Listeria monocytogenes* in RTE sliced meats.

## **2. Materials and methods**

### *2.1. Product manufacture*

Fresh trimmed ham muscles from the inside (*semimembranosus*) and knuckle (*rectus femoris*, *vastus intermedius*, *vastus lateralis*, and *vastus medialis*), as well as fresh trimmed turkey breast (*pectoralis major* and *pectoralis minor*) were utilized for the respective products. All raw materials were used within 2-4 days after harvest and were

ground using a 0.3175 cm diameter plate immediately before use. After grinding, meats for both turkey and ham were mixed for ca. 1 minute in a Blentech Auto Chef Silver Ribbon blender (Blentech Corp., Rohnert Park, CA.) to assure homogeneity and randomly assigned to 1 of 4 treatments. Formulations consisted of two salt concentrations (1.8% and 2.4% of formulation) and either 0 or 200 ppm sodium nitrite (with 500 ppm sodium erythorbate in the 200 ppm formula only) based on meat weight for both turkey and ham products. The remaining part of the formulations for each of the salt and nitrite treatments consisted of 1% dextrose (ADM Corn Processing, Decatur, IL), 0.4% sodium tripolyphosphate (Nutrifos O-88 - ICL Performance Products LP, St. Louis Mo.), and water/ice (to target total moisture of 77% in final product). Formulations were calculated as close as possible to equalize moisture, fat, and protein levels in both species, based on values from initial screening of raw materials, to allow assessment based on the variables of species type, salt level, and presence/absence of nitrite, independent of proximate composition.

Dry ingredients were dissolved in 85% of the water using a Lightnin mixer (Model S1UO3A, Lightnin, Rochester, NY) and additional water/ice was added to achieve a temperature of 28°F in the pickle solution. The pickle solution was then added along with the meat materials to the Blentech mixer and blended under vacuum for 20 minutes at 30 rpm. After blending, the mixed batter was held for 18 hours at 2°C and stuffed into 3.3" (8.38cm) diameter, non-permeable casings (Viscofan, Danville, IL). All items for each replication were cooked together via steam heat for 45 min. at 54°C, 45 min. at 63°C, 45 min. at 71°C, and ca. 1 hour at 80°C to an internal temperature of 74°C. After reaching final temperature, cooked products were showered with ca. 21°C water for

30 minutes and then chilled in a 1°C cooler to reach an internal temperature of <4°C within 6 hours of cooking.

Within 1 week after chilling, the casings were removed and each product treatment was sliced into 11 gram slices. Slices were stacked and bulk packaged into ~1 kg packages flushed with nitrogen. Because several studies have shown that *L. monocytogenes* growth may be affected by presence of lactic acid bacteria (Farber & Daley, 1994; Buchanan & Klawitter, 1992; Foegeding, Thomas, Pilkington, & Klaenhammer, 1992), samples were treated with high pressure at 600 MPa for 10 minutes to decrease the number of vegetative organisms potentially acquired during slicing/packaging.

## 2.2. Microbiological procedures

Each *L. monocytogenes* culture was grown overnight (18-24 hours) in tryptic soy broth (TSB) at 35°C and tested for purity on modified oxford agar (MOX) (VWR, Batavia, IL). After <1 week storage at 2°C, individual 11 gram ham or turkey slices were removed from bulk packages and repackaged into 13x29 cm packages (oxygen transmission rate = 3.5 cc/100 sq. in./day; Ultravac Solutions, Kansas City, Mo.) for inoculation. *Listeria monocytogenes* strains used for the study were ATCC 7644, NCTC 10890, ATCC 19112, ATCC 19114, and ATCC 19115 (Microbiologics, St. Cloud, MN.). Equal amounts of each strain were mixed into a common culture used for the inoculation. Based on prior testing, a count of  $10^9$  cfu/ml of *L. monocytogenes* in the overnight culture was used for calculation of further dilutions. Dilutions of the inoculum were made using sterile 0.1% peptone water to achieve three targeted levels of inoculum of  $10^5$ ,  $10^3$ , and

$10^1$  CFU/gram. Each level of inoculum was added to an 11-gram slice to achieve the targeted inoculum level. After inoculation, all samples were vacuum-sealed on a Multivac packaging machine (model A300; Multivac, Kansas City, MO.).

The inoculated samples were randomly assigned to one of two groups consisting of 1) Non-HHP samples and 2) HHP (180 seconds) treated samples. The experiment was replicated three times with microbial analyses completed in triplicate for each replication on each testing date. The samples of all treatments were stored at 4.4°C throughout the duration of the experiment. The evaluations of the non- HHP samples was conducted on days 0, 5, 7, 14, 19, 21 and 28 after inoculation, while the HHP-treated samples were evaluated at days 0, 28, 56, 91, 119, 154, and 182 after inoculation and HHP treatment.

For the non-HHP treated group, the samples were inoculated and the day 0 microbial measurements conducted for each treatment at ca. 2 hours post-inoculation. All dilutions were made by adding 99 ml of Butterfield's phosphate buffer to each 11-gram sample, stomaching, making further dilutions as needed, and then plating on MOX agar via direct plating. All analyses were run in triplicate and reported as CFU/gram, with the minimum detection limit being 10 CFU/gram. For the HHP-treated samples on day 0, the products were pressure treated (87,000 psi for 180 seconds) and microbial populations were determined in triplicate at ca. 2 hours after pressure treatment (ca. 3-4 hours after inoculation), using the same dilutions and plating procedures as the non-HHP samples. The MOX plates for all treatments were incubated for 48 hours at 35°C then examined for the presence or absence of growth. All populations were enumerated using standard methods for enumeration. All initial dilutions (1:10) of test samples were stored in snap-cap cups at 4.4°C for later enrichment of the sample, if needed. An uninoculated,

negative sample was also prepared for all treatments for each of the testing days to verify testing methods.

If no colonies were detected on MOX agar ( $<10$  CFU/gram), then enrichment of the pathogen was completed from the stored samples using USDA methods (USDA, 2009). Briefly, 25 ml of dilution sample was added to 225 ml of UVM broth and incubated for 24 hours at  $30^{\circ}\text{C}$ , then 0.1 ml of UVM broth/sample was transferred into 10 ml of Fraser broth with 0.1 ml Ferric Ammonium Citrate and incubated for 48 hours at  $35^{\circ}\text{C}$ . Tubes were evaluated for the presence (positive) or absence (negative) of a darkening color. The positive enrichment was considered as 1 log CFU/g and the negative enrichment was considered as  $-0.39$  log CFU/g (25 ml of total 110 ml sample was enriched {22%}, and 22% of original 11g slice is 2.42 g, therefore less than 1 CFU divided by 2.42 g equals  $<0.41$  CFU/gram or  $<-0.39$  log CFU/g). Enrichment samples were also streak plated onto MOX and incubated 48 hours at  $35^{\circ}\text{C}$  for confirmation. All enriched samples were recorded as either positive (darkened color) or negative (no color change). A negative sample means there was  $<0.41$  CFU/gram of the pathogen present in the sample. A positive sample means there was  $>0.41$  CFU/g, but less than the direct plate (i.e.  $<10$  CFU/g). Therefore, for the numerical count, a positive sample was assumed to be a count of 10 CFU/g and negative sample was assumed to be 0.41 CFU/g.

Along with typical growth on MOX selective media, confirmatory tests were completed using Rapid'*L.mono*<sup>™</sup> (Bio-Rad Laboratories, Marnes-la-Coquette, France).

### 2.3. High-pressure equipment and conditions

Samples that were processed under high hydrostatic pressure used a Quintus®

Type QFP 35L-600 unit (Avure Technologies, Kent, WA). Pressure treatment was carried out at 600 MPa for 3 minutes (not inclusive of come-up time of ca. 160 seconds, with an almost instantaneous depressurization) at a product temperature of 5°C and a starting vessel temperature of 17°C ( $\pm$  2°C) with water as the surrounding pressure-transmitting medium. All samples for each replication were processed together after inoculation on day 0 and then were stored at 4.4°C for the remainder of the experiment.

#### *2.4. Chemical analyses*

Samples were analyzed for proximate composition of moisture (AOAC, 1990a), crude protein (AOAC, 1993), and crude fat (AOAC, 1990b). Measurement of pH (USDA, 1993), residual nitrite (Clesceri et al. 1998), and NaCl (Clesceri et al. 1998) were also conducted.

#### *2.5. Statistical analysis*

Results were analyzed using the proc glm statement of SAS (SAS 9.2, SAS Institute Inc., Cary, NC, 2008). Product species, nitrite level and salt level were evaluated as fixed effects within each inoculation level, HHP time period, and days of shelf-life. For significant model effects ( $p < 0.05$ ), differences between treatment least squares means were determined using the lsd procedure. A total of 3 independent replications were conducted for each treatment with three samples evaluated at each day of analysis for each of the replications.

### **3. Results and discussion**

#### *3.1. No High Hydrostatic Pressure*

For the products not subjected to HHP, Table 1 shows the growth for the samples

with a 1 log level of inoculation. As expected, a significant growth of *L. monocytogenes* occurred from the initial counts on day 0 to the final count on day 28. On day 0, the uncured low salt ham and uncured high salt ham treatments had significantly lesser populations of *L. monocytogenes* than the cured low salt ham, with all other treatments being not significantly different ( $p>0.05$ ). On day 5, none of the populations in any of the treatments were significantly different ( $p>0.05$ ). On day 7 the treatment differences became evident. At day 19, all uncured items had significantly greater ( $p<0.05$ ) populations of *L. monocytogenes* than all cured items. This confirms previous observations that nitrite slows, but does not stop, the growth of *L. monocytogenes* (Grau & Vanderlinde, 1992; Duffy et al., 1994).

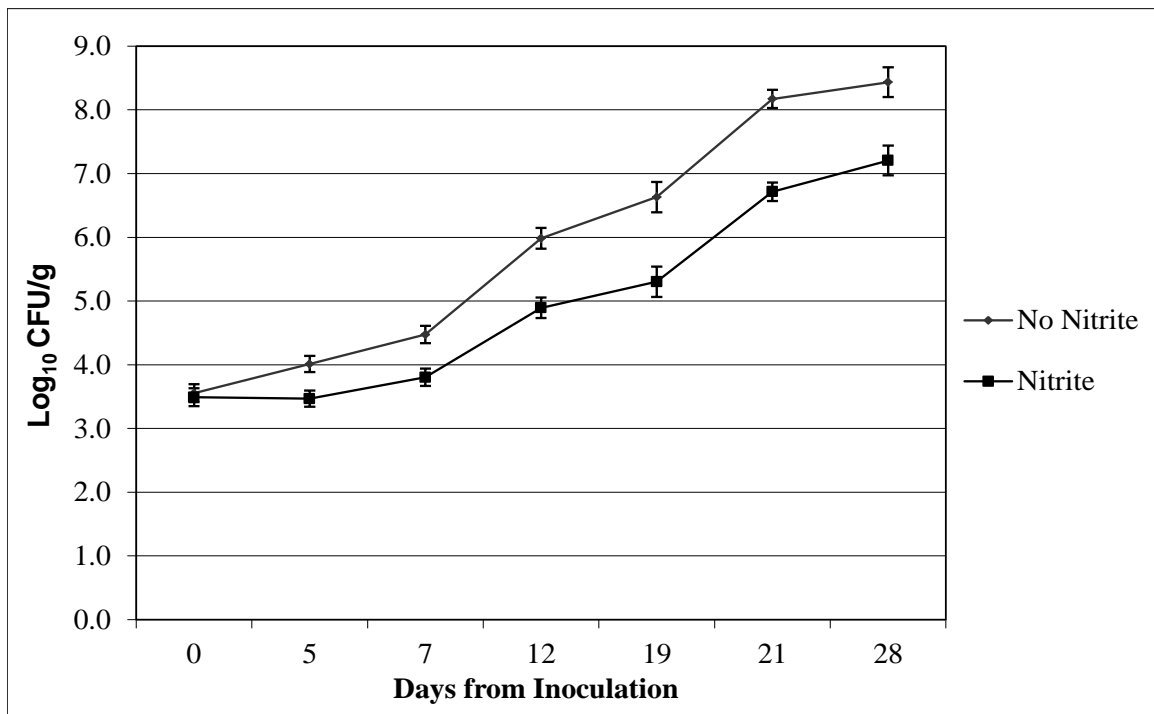
**Table 1.** Least squares means by treatment after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^1$  CFU/g, and with non-HHP treatment.

	Day 0	Day 5	Day 7	Day 12	Day 19	Day 21	Day 28
Ham - Uncured Low Salt	1.28 <sup>a</sup>	1.78 <sup>a</sup>	2.43 <sup>b</sup>	4.76 <sup>cd</sup>	6.49 <sup>b</sup>	7.10 <sup>abc</sup>	8.27 <sup>bc</sup>
Ham - Uncured High Salt	1.22 <sup>a</sup>	1.88 <sup>a</sup>	2.07 <sup>ab</sup>	4.29 <sup>bcd</sup>	6.18 <sup>b</sup>	6.54 <sup>bc</sup>	7.97 <sup>b</sup>
Ham - Cured Low Salt	1.66 <sup>b</sup>	1.44 <sup>a</sup>	1.84 <sup>ab</sup>	3.20 <sup>abc</sup>	4.59 <sup>a</sup>	5.41 <sup>abc</sup>	7.25 <sup>b</sup>
Ham - Cured High Salt	1.44 <sup>ab</sup>	1.52 <sup>a</sup>	1.67 <sup>ab</sup>	2.88 <sup>ab</sup>	4.35 <sup>a</sup>	4.83 <sup>a</sup>	7.66 <sup>b</sup>
Turkey - Uncured Low Salt	1.32 <sup>ab</sup>	1.85 <sup>a</sup>	2.39 <sup>b</sup>	5.07 <sup>d</sup>	6.65 <sup>b</sup>	7.00 <sup>bc</sup>	8.05 <sup>b</sup>
Turkey - Uncured High Salt	1.39 <sup>ab</sup>	1.64 <sup>a</sup>	2.31 <sup>b</sup>	4.65 <sup>cd</sup>	6.03 <sup>b</sup>	6.55 <sup>bc</sup>	7.98 <sup>b</sup>
Turkey - Cured Low Salt	1.30 <sup>ab</sup>	1.27 <sup>a</sup>	1.82 <sup>ab</sup>	3.08 <sup>abc</sup>	4.51 <sup>a</sup>	5.10 <sup>ab</sup>	6.37 <sup>ab</sup>
Turkey - Cured High Salt	1.45 <sup>ab</sup>	1.33 <sup>a</sup>	1.28 <sup>a</sup>	2.40 <sup>a</sup>	3.66 <sup>a</sup>	4.32 <sup>a</sup>	5.44 <sup>a</sup>
Std. Error	0.12	0.27	0.30	0.53	0.45	0.56	0.59

<sup>a,b,c,d</sup> Means with different superscripts show differences among means within a column ( $p<0.05$ )



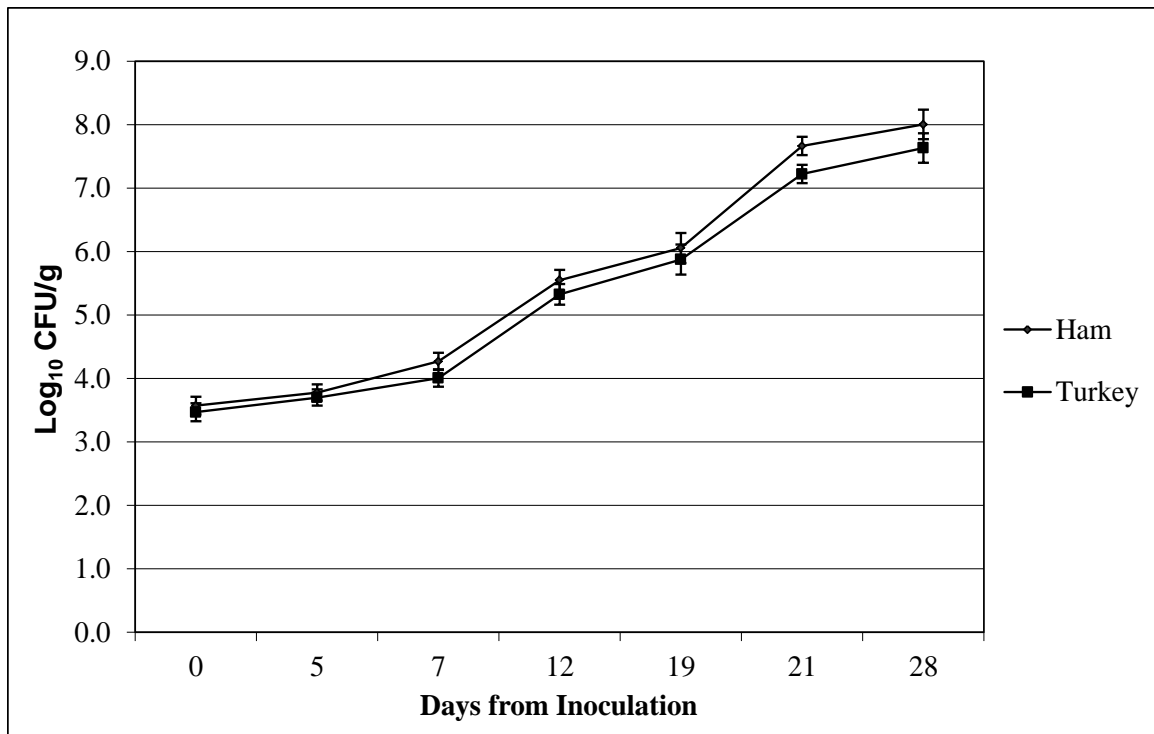
For the non-HHP samples with a 3 log inoculation, there were no significant differences at day 0, but by day 21, all uncured items showed greater populations of *L. monocytogenes* compared to all of the cured items (data not shown). The pooled LS means for nitrite and no-nitrite treatments with a 3 log inoculation are shown in Figure 1. The pooled LS means show a significantly reduced ( $p<0.05$ ) number of *L. monocytogenes* for samples containing sodium nitrite compared to those with no nitrite for day 5 through day 28 of the evaluation. Residual nitrite concentrations for the nitrite-containing samples averaged 74 ppm on day 0 and 52 ppm on day 28. For the samples without added sodium nitrite, the analyses showed  $<1$ ppm nitrite at both 0 and 28 days.



**Fig. 1.** Least squares means of treatments with and without added sodium nitrite after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g, and with non-HHP treatment.

The pooled LS means for turkey and ham with 3 log inoculation are shown in

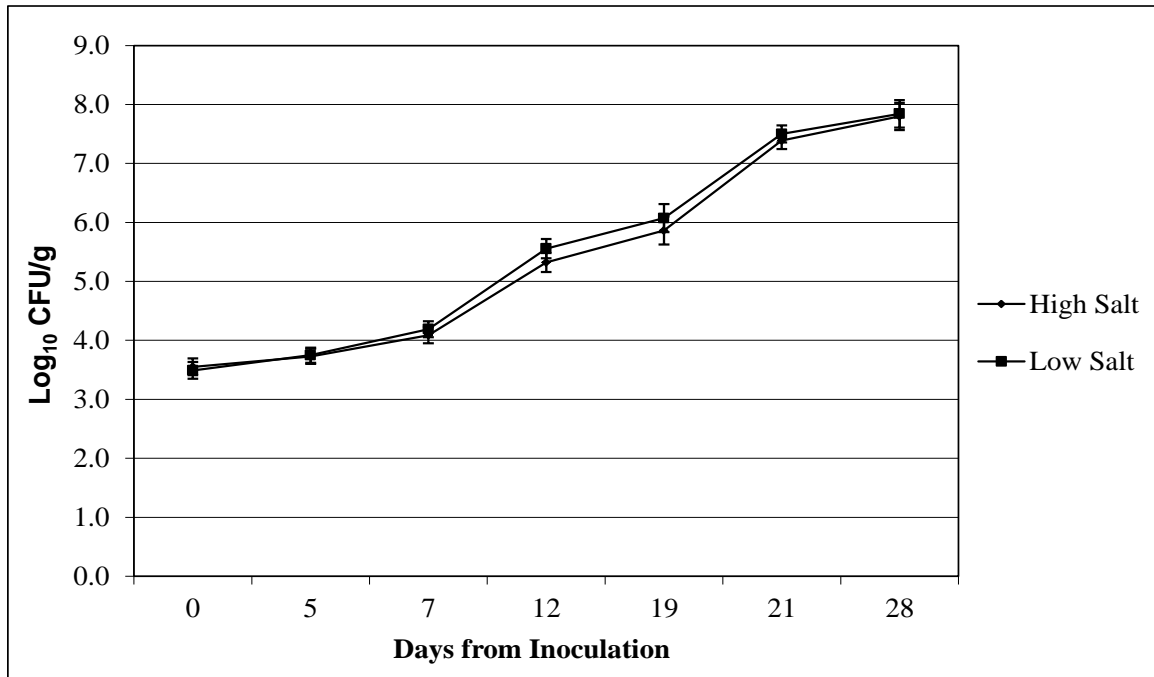
Figure 2. There were no significant differences ( $p>0.05$ ) in the *L. monocytogenes* population for any of the days, with the exception of day 21, in which the ham sample had a significantly greater count of *L. monocytogenes* than turkey ( $p<0.05$ ). This supports the findings of Duffy et al. (1994) that the species of meat used in processed meats had no effect on growth rate of *L. monocytogenes*.



**Fig. 2.** Least squares means by species (turkey and ham) after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g, and with non-HHP treatment.

The pooled LS means for low salt and high salt for the 3 log inoculations are shown in Figure 3. There was no significant difference ( $p>0.05$ ) in *L. monocytogenes* population for the two concentrations of salt at any of the evaluation days. The product analyses shown in Table 2 indicate that the finished product salt concentrations were very similar to the target levels of 1.80% in the low salt formulations and 2.40% in the high salt formulations. The non-HHP 5 log inoculations showed very similar treatment effects

for salt and species, but over a shorter duration, taking 7-14 less days to reach peak counts (data not shown).



**Fig. 3.** Least squares means by salt level after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g, and with non-HHP treatment.

**Table 2.** Average product moisture, fat, and protein concentration ; moisture:protein by species, and average salt and brine strength by salt concentration and species.

	Ham	Turkey	Std. Error
Moisture	76.77% <sup>b</sup>	76.21% <sup>a</sup>	0.11
Protein	18.23% <sup>a</sup>	19.50% <sup>b</sup>	0.09
Fat	1.34% <sup>b</sup>	0.97% <sup>a</sup>	0.06
pH	6.40 <sup>b</sup>	6.33 <sup>a</sup>	0.01
Moisture: protein ratio	4.21 <sup>b</sup>	3.91 <sup>a</sup>	0.09
% Salt (high salt formula)	2.35% <sup>a</sup>	2.34% <sup>a</sup>	0.02%
% Salt (low salt formula)	1.80% <sup>a</sup>	1.78% <sup>a</sup>	0.02%
Brine strength (high salt)	2.97% <sup>a</sup>	2.99% <sup>a</sup>	0.02%
Brine strength (low salt)	2.29% <sup>a</sup>	2.27% <sup>a</sup>	0.02%

<sup>a,b</sup> Means with different superscripts show differences among species means ( $p < 0.05$ )

### 3.2. With High Hydrostatic Pressure

For the samples subjected to high pressure, a reduction in *L. monocytogenes* of about 3.85 - 4.35 log CFU/g was achieved immediately after high pressure and there were no significant ( $p>0.05$ ) formulation or species differences (Table 3).

**Table 3.** Log<sub>10</sub> reduction in plate count of *L. monocytogenes* after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^5$  CFU/g. (S.E. = 0.39)

Treatments:	Log <sub>10</sub> Reduction (CFU/g)
Ham - Uncured Low Salt	4.35 <sup>a</sup>
Ham - Uncured High Salt	4.29 <sup>a</sup>
Ham - Cured Low Salt	4.15 <sup>a</sup>
Ham - Cured High Salt	4.07 <sup>a</sup>
Turkey - Uncured Low Salt	4.10 <sup>a</sup>
Turkey - Uncured High Salt	4.06 <sup>a</sup>
Turkey - Cured Low Salt	4.29 <sup>a</sup>
Turkey - Cured High Salt	3.85 <sup>a</sup>

For the 3 log inoculation treatments, immediately after high pressure treatment application, all treatments had a reduction in *L. monocytogenes* numbers to below the detection limit of 1.0 log CFU/g. All treatments then remained below the detection limit until 154 days of storage following HHP (Table 4). Least squares means for *L. monocytogenes* numbers averaged above the detection limit of 1 log CFU/g at 154 days post-HHP for uncured high salt ham and at 182 days post-HHP for uncured high salt ham and uncured low salt turkey. This growth was observed only in replication three of the experiment, which had an inoculation level of 3.42 logs – slightly greater than the target

of 3.0 log CFU/g. The average log CFU/g values for day 0 non-HHP treatments for replications 1, 2, and 3 were 3.42, 3.10, and 4.04, respectively, with the third replication having a significantly greater ( $p<0.05$ ) number of *L. monocytogenes* on day 0 than the other two replications.

**Table 4.** Least squares means by treatment after inoculation with a 5-strain mixed culture of *L. monocytogenes* at  $10^3$  CFU/g followed by HHP treatment at 600 MPa for 3 minutes

	Pre-HP	Day 0	Day 28	Day 56	Day 91	Day 119	Day 154	Day 182
Ham - Uncured Low Salt	3.54 <sup>a</sup>	-0.23 <sup>a</sup>	0.54 <sup>a</sup>	0.23 <sup>a</sup>	0.23 <sup>a</sup>	0.38 <sup>a</sup>	-0.08 <sup>a</sup>	0.22 <sup>a</sup>
Ham - Uncured High Salt	3.69 <sup>a</sup>	-0.08 <sup>a</sup>	0.38 <sup>a</sup>	0.70 <sup>a</sup>	0.54 <sup>a</sup>	0.38 <sup>a</sup>	1.85 <sup>a</sup>	2.38 <sup>a</sup>
Ham - Cured Low Salt	3.50 <sup>a</sup>	-0.08 <sup>a</sup>	-0.08 <sup>a</sup>	0.08 <sup>a</sup>	0.38 <sup>a</sup>	-0.08 <sup>a</sup>	-0.08 <sup>a</sup>	0.07 <sup>a</sup>
Ham - Cured High Salt	3.56 <sup>a</sup>	-0.23 <sup>a</sup>	0.08 <sup>a</sup>	-0.08 <sup>a</sup>	-0.23 <sup>a</sup>	-0.39 <sup>a</sup>	0.13 <sup>a</sup>	-0.08 <sup>a</sup>
Turkey - Uncured Low Salt	3.52 <sup>a</sup>	0.13 <sup>a</sup>	0.07 <sup>a</sup>	0.07 <sup>a</sup>	0.07 <sup>a</sup>	0.23 <sup>a</sup>	-0.39 <sup>a</sup>	2.12 <sup>a</sup>
Turkey - Uncured High Salt	3.46 <sup>a</sup>	-0.39 <sup>a</sup>	-0.39 <sup>a</sup>	-0.08 <sup>a</sup>	0.07 <sup>a</sup>	0.23 <sup>a</sup>	0.90 <sup>a</sup>	0.08 <sup>a</sup>
Turkey - Cured Low Salt	3.41 <sup>a</sup>	-0.39 <sup>a</sup>	-0.23 <sup>a</sup>	-0.23 <sup>a</sup>	-0.23 <sup>a</sup>	-0.08 <sup>a</sup>	-0.23 <sup>a</sup>	-0.39 <sup>a</sup>
Turkey - Cured High Salt	3.49 <sup>a</sup>	-0.08 <sup>a</sup>	-0.23 <sup>a</sup>	-0.08 <sup>a</sup>	-0.23 <sup>a</sup>	0.08 <sup>a</sup>	-0.23 <sup>a</sup>	0.62 <sup>a</sup>
Std. Error	0.12	0.12	0.27	0.30	0.53	0.45	0.56	0.59

<sup>a</sup> Means with the same superscript show no differences among means in a column ( $p>0.05$ )

There were no statistically significant differences at either 154 or 182 days between any of the individual treatments after HHP. There was however a significant interaction between nitrite and salt ( $p<0.05$ ) at 154 days. The LS mean for no nitrite/high salt was 2.63, which was significantly greater ( $p<0.05$ ) than the LS means for no nitrite/low salt of -0.074, and 200 ppm nitrite/low salt of -0.028, respectively. There were no significant interaction effects for the 182 day evaluation.

**Table 5.** Number of samples showing visible growth of *L. monocytogenes* at 154 and 182 days of shelf life after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g followed by HHP treatment at 600 MPa for 3 minutes (three replications with three measurements at each replication).

<b>Treatments:</b>	<b>Day 154</b> Counts > $10^1$ CFU/g	<b>Day 182</b> Counts > $10^1$ CFU/g
Ham - Uncured Low Salt	0 of 9	1 of 9
Ham - Uncured High Salt	2 of 9	3 of 9
Ham - Cured Low Salt	0 of 9	0 of 9
Ham - Cured High Salt	1 of 9	0 of 9
Turkey - Uncured Low Salt	0 of 9	3 of 9
Turkey - Uncured High Salt	1 of 9	0 of 9
Turkey - Cured Low Salt	0 of 9	0 of 9
Turkey - Cured High Salt	0 of 9	2 of 9

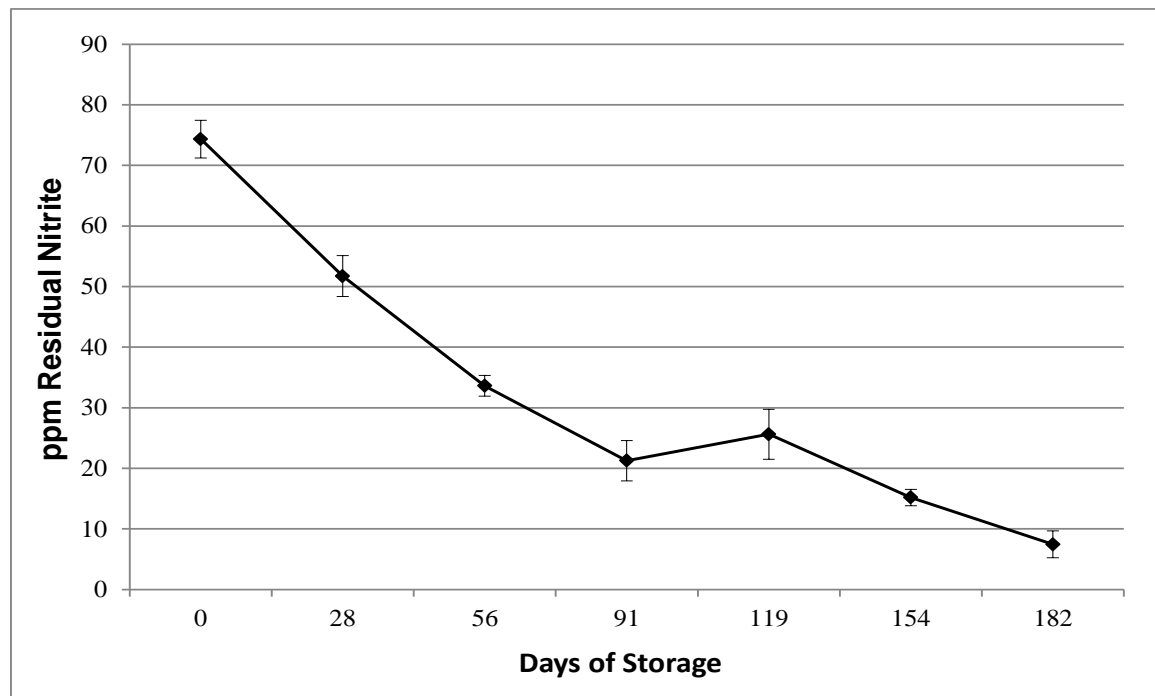
Table 5 shows the number of samples for each treatment that demonstrated growth (above detection level of  $1.0 \log$  CFU/g) out of the total 9 samples evaluated for each treatment (3 replications with 3 measures each) at 154 or 182 days. The samples that showed growth seemed to be random with no clear relationship to nitrite, salt, or species. This somewhat erratic growth mentioned before may be due to the greater inoculation level of replication 3. The level of inoculum in the third replication was probably close to the maximum level for eradication of *L. monocytogenes* via HHP, thus permitting *L. monocytogenes* survivors in some packages that, after time for repair, were able to grow to high levels. Ritz, Pilet, Jugiau, Rama, and Federighi (2006) and Bozoglu, Alpas, and Kaletunc (2004) found that *L. monocytogenes* cells exposed to high pressure were able to repair and grow over time, with lesser numbers of *L. monocytogenes*

observed at greater pressure levels and reduced storage temperatures. Bowman, Bittencourt, and Ross (2008) pointed out that the septal ring in the *L. monocytogenes* cell membrane is important in cell replication and may be damaged by HHP, but if able to be repaired could result in regeneration and renewed growth of the pathogen.

For the 1 log inoculations followed by HHP, all treatments resulted in no growth throughout the 182 day storage period of the study (data not shown).

### 3.3. Chemical analyses

The average moisture, fat, and protein analyses by species are shown in Table 2. The turkey breast raw material had a significantly greater ( $p < 0.05$ ) concentration of protein (19.35%) compared to the ham items (18.15%). There were also significant differences ( $p < 0.05$ ) in the moisture, fat, moisture: protein, and pH between species, but the differences were within a fairly tight range.



**Fig. 4.** Average residual nitrite level of formulations with sodium nitrite added (uninoculated samples with 10 minute HHP treatment).

Average residual nitrite values for the treatments that contained added sodium nitrite are shown in Figure 4. The formulated level of 200 ppm nitrite decreased to 74 ppm of residual nitrite after cooking and slicing. The residual nitrite level then decreased further over the shelf life of the product, and is negatively correlated with time, with a correlation value of -0.933. All treatments with added sodium nitrite still had detectable levels of residual nitrite at the end of the 182 day study. Duffy et al. (1994) also showed that levels of residual nitrite decreased over time but that greater concentrations of residual nitrite increased inhibition of *L. monocytogenes* in vacuum-packaged ready-to-eat meats.

#### **4. Conclusions**

This study suggested that there were no differences in the growth of *L. monocytogenes* due to species, throughout the 182-day sampling period. Other studies (Mujahid, Pechan, & Wang, 2008; Glass & Doyle, 1989; Farber & Daley, 1994a; Lin et al., 2006; Lianou et al., 2007b; Pal, Labuza, & Diez-Gonzalez, 2008) have reported that *L. monocytogenes* grew very readily in turkey, but unlike those studies, this experiment used formulations that were as similar as possible in proximate composition for both the turkey and ham products. The present study also showed that, for the formulations evaluated without use of HHP, the absence of sodium nitrite allowed for increased growth of *L. monocytogenes*. The use of HHP greatly reduced counts of *L. monocytogenes* by more than 3 log CFU/g, but when the inoculation level was > ~3.4 log CFU/g, some survivors were able to repair and grow at >120 days after HHP treatment when stored at 4.4°F.



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### **CHAPTER 3 - Effects of sodium nitrite and concentration of pre-converted vegetable juice powder on growth of *Listeria monocytogenes* in RTE sliced ham with and without high hydrostatic pressure**

A paper to be submitted to the journal *Meat Science*

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#### **Abstract**

The objective of this study was to determine the effect of the source of added nitrite and high hydrostatic pressure (HHP) on the growth of *Listeria monocytogenes*. Use of 600 MPa HHP for 3 minutes resulted in an immediate 3.5-4.2 log CFU/g reduction in *Listeria monocytogenes* numbers, while use of 400 MPa HHP (3 min.) gave less than 1 log CFU/g reduction. Use of a conventional level of sodium nitrite (200 ppm) was not different in *Listeria monocytogenes* growth from use of 50 or 100 ppm pre-converted nitrite from a natural source. Use of 150 or 200 ppm pre-converted natural nitrite showed increased *Listeria monocytogenes* growth at beyond 56 days of storage after HHP, possibly due to increased pH in the finished product. Instrumental color, residual nitrite, and residual nitrate levels for cured (sodium nitrite and natural nitrite sources) and uncured ham items are reported and discussed.

#### **1. Introduction**

Scallan et al., (2011) reported that foodborne pathogens caused an average of 9.4 million illnesses and 1,351 deaths per year over the past decade in the United States. Raw meat materials have the capability of supporting the survival and growth of *Listeria monocytogenes* (Wimpfheimer, Altman, & Hotchkiss, 1990; Chasseignaux, Toquin,

Ragimbeau, Salvat, Colin, & Ermel, 2001; Doi, Ono, Saitoh, Ohtsuka, Shibata, & Masaki, 2003), but heat processing methods used by manufacturers of ready-to-eat (RTE) meats are sufficient to eliminate *L. monocytogenes* from ready-to-eat products (Sauders & Wiedmann, 2007; Kornacki & Gurtler, 2007).

*L. monocytogenes* is recognized to be a pathogen of upmost concern for ready-to-eat meat items due to the potential risk of contamination during post-lethality handling in processes such as slicing or packaging (Hwang & Tamplin, 2007; Kornacki & Gurtler, 2007). Lin et al. (2006) determined that *L. monocytogenes* coming into contact with a commercial slicer blade, or other machinery parts that contact the meat surface, could subsequently be transferred onto the surface of ready-to-eat sliced meats. *L. monocytogenes* has a potential to cause a high level of mortality to the immunocompromised, including the elderly and to unborn fetuses (Vazquez-Boland et al., 2001; Painter & Slutsker, 2007; Skogberg et al., 1992).

Sodium nitrite has been shown to be an inhibitor of *L. monocytogenes* in ready-to-eat meat items (Buchanan, Stahl, & Whiting, 1989; Buchanan & Phillips, 1990; McClure, Kelly, & Roberts, 1991; Grau & Vanderlinde 1992; Duffy, Vanderlinde, & Grau, 1994; Farber & Daley, 1994). However, Schlyter, Glass, Loeffelholz, Degnan, and Luchansky (1993) found that levels of 30 ppm of sodium nitrite added after cooking was insufficient to inhibit growth of *L. monocytogenes*.

There has been a dramatic growth of foods that are labeled natural, minimally-processed, or preservative-free over the past several years. It is estimated that sales of natural foods in the United States will reach \$30 billion by 2014 (Nunes, 2011). One category in which this growth has occurred is in ready-to-eat meat items labeled as

“Natural” or “Preservative-free”, leading to growth of a new category of uncured or “naturally-cured” processed meats (Sebranek & Bacus, 2007; Sindelar, Cordray, Sebranek, Love, & Ahn, 2007; Sindelar, Cordray, Olson, Sebranek, & Love, 2007). Since sodium nitrite is considered a chemical preservative by USDA, it is not allowed to be used in items that are labeled Natural or Preservative-free (FSIS, 2006). Many of these items either have no nitrite or they utilize natural sources of nitrate, such as various vegetable materials, that are converted to nitrite via microbial fermentation during the cooking process of the meat item (Sebranek & Bacus, 2007; Sindelar et al., 2007b; Sindelar et al., 2007a). Nitrate-reducing bacterial starter cultures can also be added directly to the nitrate-containing vegetable juice before it is added to the meat, and in fact these “pre-converted” materials with nitrite are now commercially-available to provide improved process efficiency for manufacturing of items with a cured appearance, but meet the USDA definition for natural products (Sebranek & Bacus, 2007; Xi, Sullivan, Jackson, Zhou, & Sebranek, 2012; Krause, Sebranek, Rust, & Mendonca, 2011).

Sindelar et al. (2007b) found that uncured, no-nitrite added products at a low concentration of vegetable juice powder (0.20%) had similar sensory scores to products conventionally-cured using sodium nitrite. However, the residual nitrite levels of these items were less than the conventionally-cured products and thereby if contaminated with *L. monocytogenes* during post-lethality processing, had a potential for greater numbers of organisms during shelf life. In a challenge study, Schrader (2010) found that ready-to-eat meat items that contained celery juice powder as a source of added sodium nitrite or sodium nitrate were not able to retard growth of *L. monocytogenes* as effectively as conventionally cured products for a period of 35 days when stored at 10°C.

High hydrostatic pressure (HHP) has been used for food items to extend shelf life and minimize the risk of bacterial pathogens (Martin, Barbosa-Canovas, & Swanson, 2002; Patterson, 2005). Processing of foods under high hydrostatic pressure has helped food processors to meet growing consumer demands for natural, minimally-processed, and preservative-free products, while maintaining consistent sensory characteristics over an extended shelf life, and still assuring product safety (Patterson, 2005; Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008; Yordanov & Angelova, 2010). Several review papers have been written on the effects of high hydrostatic pressure on foods, with particular emphasis on the capability for suppression of bacterial pathogens in ready-to-eat foods (Smelt, 1998; Farkas & Hoover, 2000; Martin, et al., 2002; Patterson, 2005; Balasubramaniam, Farkas, & Turek, 2008; Aymerich, Picouet, & Monfort, 2008; Considine et al., 2008; Yordanov & Angelova, 2010).

The purpose of this study was to determine the extent to which nitrite source and HHP influence *Listeria monocytogenes* growth in a sliced RTE ham. The study will focus on the inhibition of *L. monocytogenes* by sodium nitrite in a “conventional” USDA standard of identity ham formulation compared to use of a vegetable juice powder (natural nitrate source) that has been pre-converted via nitrate-reducing bacteria to provide a commercially-available natural source of nitrite for a natural sliced ham item. Testing of *L. monocytogenes* growth in both types of formulations will be evaluated both with and without the use of post-lethality HHP processing at 400 MPa and 600 MPa.

## **2. Materials and methods**

### *2.1. Product manufacture*



Fresh trimmed ham muscles from the inside (*semimembranosus*) and knuckle (*rectus femoris*, *vastus intermedius*, *vastus lateralis*, and *vastus medialis*) were used for manufacture of hams. All raw materials were used within 2-4 days after harvest and were ground using a 0.3175 cm diameter plate immediately before use. After grinding, meats were mixed in a Blentech Auto Chef Silver Ribbon blender (Blentech Corp., Rohnert Park, CA.) for ca. 1 minute to assure homogeneity and randomly assigned to treatments. Formulation information is listed in Table 1.

**Table 1.** Ingredient breakdown for natural or conventional formulas.

	Conventional formulations		Natural formulations	
	NaNO <sub>2</sub> 100	NaNO <sub>2</sub> 200	Pre-converted	VJP
Salt	2.40%	2.40%	2.40%	2.40%
Sugar	1.00%	1.00%	1.00%	1.00%
Ham	81.97%	81.97%	81.97%	81.97%
Water	14.18%	14.17%	14.45%***	14.27%
Sodium phosphate	0.40%	0.40%	-	-
Sodium nitrite*	100 ppm	200 ppm	-	-
Sodium erythorbate*	500 ppm	500 ppm	-	-
VegStable 506*	-	-	**0.22% (50ppm) 0.44% (100 ppm) 0.67% (150 ppm) 0.89% (200 ppm)	-
VegStable 502	-	-	-	0.44%

\* percentage of total meat weight

\*\*four different formulas at stated target of 50, 100, 150, & 200 mg/kg VegStable 506 as a percentage of total meat weight

\*\*\*water shown is for 50 Natural formula – water was reduced by the corresponding amount of VegStable 506 added to 100, 150, and 200 Natural formulations on basis of total formulation percentage

All formulations consisted of 1% sugar (United Sugars, Bloomington, MN.) and 2.4% salt (sodium chloride - Morton Salt, Chicago, IL.). Conventionally cured products contained 0.4% sodium tripolyphosphate (ICL Performance Products LP, St. Louis, MO.), 500 ppm sodium erythorbate, and either 100 ppm or 200 ppm sodium nitrite, based on raw meat weight. Products without added sodium nitrite, but containing a “natural” source of nitrite (vegetable juice powder with nitrate pre-converted via addition of starter culture to convert nitrate to nitrite) were formulated using VegStable 506 (Florida Food Products, Eustis, FL.) to contain 50, 100, 150, or 200 ppm of formulated nitrite based on raw meat weight. Based on analysis, VegStable 506 had 22,500 ppm of nitrite, so the four treatments with natural nitrite at 50, 100, 150, and 200 ppm contained 0.22%, 0.44%, 0.67%, and 0.89% VegStable 506. A control with no added nitrite/nitrite and an additional control using a nitrate-containing vegetable juice powder (VegStable 502 – Florida Food Products, Eustis, FL.) at 0.44% of meat weight were also included in the test. Water was added as the remaining ingredient to give a total of 22% total added ingredients based on the raw meat weight.

Dry ingredients were dissolved in ca. 85% of the water using a Lightnin mixer (Model S1UO3A, Lightnin, Rochester, NY) and additional water/ice was added to achieve a temperature of 28°F in the pickle solution. The pickle solution was then added along with the meat materials to the Blentech mixer and blended under vacuum for 20 minutes at 30 rpm. After blending, the mixed batter was stuffed into 8.38 cm diameter, non-permeable casings (Viscofan, Danville, IL) and held for 18 hours at 2°C before cooking. All items for each replication were cooked together via steam heat for 45 min. at

54°C, 45 min. at 63°C, 45 min. at 71°C, and ca. 1 hour at 80°C to an internal temperature of 74°C. After reaching final temperature, cooked products were showered with ca. 21°C water for 30 minutes and then chilled in a 1°C cooler to reach an internal temperature of <4°C within 6 hours of cooking.

Within 1 week after chilling, the casings were removed and each of the product treatments were sliced into 11 gram slices. Slices were stacked and bulk packaged into ~2 pound vacuum packages (Curwood, Inc., Oshkosh, WI). Because studies have shown that *L. monocytogenes* growth may be affected by lactic acid bacteria (Farber & Daley, 1994; Buchanan & Klawitter, 1992; Foegeding, Thomas, Pilkington, & Klaenhammer, 1992), samples were treated with high pressure at 600 MPa for 10 minutes to decrease the number of vegetative organisms potentially acquired during slicing/packaging.

## 2.2. Microbiological procedures

Each *L. monocytogenes* culture was grown overnight (18-24 hours) in TSB at 35°C and each culture was tested for purity on modified oxford agar (MOX) (VWR, Batavia, IL). After <1 week storage at 2°C, individual 11g ham slices were repackaged into 20 by 35 cm packages (O<sub>2</sub> transmission rate = 0.5 cc/100 sq. in./day; Cryovac – Sealed Air Corp., Duncan, SC.) for inoculation. *Listeria monocytogenes* strains used for the study were ATCC 7644, NCTC 10890, ATCC 19112, ATCC 19114, and ATCC 19115 (Microbiologics, St. Cloud, MN.). Equal amounts of each strain were mixed into a common mixed culture used for the inoculation. Based on prior testing a count of 10<sup>9</sup> cfu/ml of *L. monocytogenes* in the overnight cultures was used for calculation of further dilutions. Dilutions of the inoculum were made using sterile 0.1% peptone water to

achieve two targeted levels of inoculum of  $10^5$  and  $10^3$  CFU/gram. Each level of inoculum was added to an 11-gram slice to achieve the targeted level. After inoculation, all samples were vacuum-sealed on a Multivac packaging machine (model A300; Multivac, Kansas City, MO.).

The inoculated samples were randomly assigned to one of three groups consisting of 1) No-HHP samples, 2) 600 MPa HHP (180 seconds) treated samples, or 3) 400 MPa HHP (180 seconds) treated samples. The experimental treatments were independently replicated either two times (No Nitrite, VJP, and  $\text{NaNO}_2$ 200) or three times (Natural treatments and  $\text{NaNO}_2$ 100), with microbial analyses completed in triplicate for each replication on each testing date. The samples of all treatments were stored at  $4.4^\circ\text{C}$  throughout the duration of the experiment. For the  $10^5$  inoculum level, evaluations were performed only on day 0 to determine *L. monocytogenes* population differences before and after HHP treatment. For the  $10^3$  inoculum level, the evaluations of the no-HHP samples were conducted on days 0, 5, 7, 14, 19, and 21 after inoculation, while the 400 MPa HHP-treated samples were evaluated at days 0, 14, 28, 42, 56, and 91 after inoculation, and the 600 MPa HHP-treated samples were evaluated at days 0, 14, 28, 42, 56, 91, 119, 154, and 182 after inoculation.

For the no-HHP treated group, the samples were inoculated and the day 0 microbial measurements conducted for each treatment at ca. 2 hours after being inoculated. All dilutions were made by adding 99 ml of Butterfield's phosphate buffer to each 11-gram sample, stomaching, making further dilutions as needed, and then plating on MOX agar via direct plating. All counts were run in triplicate for each replication, with each measurement converted to a logarithmic scale and averaged to give log

CFU/gram for each replication. The minimum detection limit for *L. monocytogenes* was 10 CFU/gram. For the HHP-treated samples on day 0, the products were pressure treated (600 MPa or 400 MPa for 180 seconds) and microbial populations were determined in triplicate at ca. 1-2 hours after pressure treatment (ca. 2-3 hours after inoculation), using the same dilution and plating procedures as the no-HHP samples. The MOX plates for all treatments were incubated for 48 hours at 35°C then examined for the presence or absence of growth. All populations were enumerated using standard methods for enumeration. All initial dilutions (1:10) of test samples were stored in snap-cap cups at 4.4°C for later enrichment of the sample, if needed. An uninoculated, negative sample was also prepared for all treatments for each of the testing days.

If no colonies were detected on MOX agar (<10 CFU/gram), then enrichment of the stored sample was completed using USDA methods (USDA, 2009). Briefly, 25 ml of dilution sample was added to 225 ml of UVM broth and incubated for 24 hours at 30°C, then 0.1 ml of UVM broth/sample was transferred into 10 ml of Fraser broth with 0.1 ml Ferric Ammonium Citrate and incubated for 48 hours at 35°C. Tubes were evaluated for the presence (positive) or absence (negative) of a darkening color. The positive enrichment was considered as 1 log CFU/g and the negative enrichment was considered as -0.39 log CFU/g (25 ml of total 110 ml sample was enriched {22%}, and 22% of original 11g slice is 2.42 g, therefore less than 1 CFU divided by 2.42 g equals <0.41 CFU/gram or <-0.39 log CFU/g). Enrichment samples were also streak plated onto MOX and incubated 48 hours at 35°C for confirmation. All enriched samples were recorded as either positive (darkened color) or negative (no color change) result. A negative sample means there was <0.41 CFU/gram of the pathogen present in the sample.

A positive sample means there was >0.41 CFU/g, but less than the direct plate (i.e. <10 CFU/g). Therefore, for the numerical count, a positive sample was assumed to be a count of 10 CFU/g and negative sample was assumed to be 0.41 CFU/g.

Along with typical growth on MOX selective media, confirmatory tests were completed using Rapid'*L.mono*<sup>TM</sup> (Bio-Rad Laboratories, Marnes-la-Coquette, France).

### *2.3. High-pressure equipment and conditions*

Samples that were processed under high hydrostatic pressure used a Quintus® Type QFP 35L-600 unit (Avure Technologies, Kent, WA). Pressure treatment was carried out at 600 MPa for 3 minutes (not inclusive of come-up time of ca. 160 seconds, with an almost instantaneous depressurization) or at 400 MPa for 3 minutes (not inclusive of come-up time of ca. 90 seconds, with an almost instantaneous depressurization) at a product temperature of 5°C and a starting vessel temperature of 17°C (± 2°C) with water as the surrounding pressure-transmitting medium. All samples for each replication were processed together after inoculation on day 0 and then were stored at 4.4°C for the remainder of the experiment.

### *2.4. Chemical analyses*

Samples were analyzed for proximate composition of moisture (AOAC, 1990a), crude protein (AOAC, 1993), and crude fat (AOAC, 1990b). Measurement of residual nitrite (Clesceri et al. 1998a), residual nitrate (Clesceri et al. 1998b), and NaCl (Clesceri et al. 1998c) were also conducted.

### *2.5. Color analysis*

Objective color measurements (L\*, a\*, b\*) were collected on ham slices at ca. 40

days after slicing and with 10 minute application of HHP using a Minolta colorimeter (Minolta CR400 Chromameter, Konica Minolta, New Jersey) utilizing a D65 illuminant, a 0 degree observer and an 8 mm aperture. A white tile provided by the manufacturer was used to calibrate the instrument. An average value for L\*, a\*, and b\* from 20 slices in each replication was used for analysis.

## *2.6. Statistical analysis*

Results were analyzed using the proc glm statement of SAS (SAS 9.2, SAS Institute Inc., Cary, NC, 2008). For significant model effects ( $p < 0.05$ ), differences between treatment least squares means were determined using the lsd procedure. For the 3 log inoculation analysis, log reduction was analyzed within HHP treatment. The fixed effects of formulation, days of shelf-life, and replication plus the interaction of formulation \* days of shelf life were used in the analysis. For the analysis of pH and proximate composition of moisture, fat, protein, and salt, the formulation and replication effects were fixed in the model. For residual nitrite/nitrate, formulation, days of shelf-life, formulation \* days of shelf life interaction, and replication were fixed effects. For the analysis of the change in nitrite due to HHP, the difference in nitrite concentration before HHP and after HHP was calculated and analyzed using the fixed effects of formulation and HHP level.

## **3. Results and discussion**

Items that were not treated via high hydrostatic pressure (no-HHP) exhibited significant growth of *L. monocytogenes* ( $p < 0.05$ ) from day 0 through day 21. Table 2 shows the means of treatments with a 3 log level of inoculation and no HHP. All

treatments were not significantly different ( $p>0.05$ ) in the level of *L. monocytogenes* until day 12. The treatment with 200 ppm of sodium nitrite ( $\text{NaNO}_2$  200) had significantly less *L. monocytogenes* ( $p<0.05$ ) on days 12 and 14 than the VJP, 50 Natural, 100 Natural, and 150 Natural treatments. Also at days 12 and 14, the  $\text{NaNO}_2$ 100 treatment had significantly fewer numbers of *L. monocytogenes* ( $p<0.05$ ) than the 50 Natural and 100 Natural treatments. The  $\text{NaNO}_2$  200 treatment also had significantly less *L. monocytogenes* ( $p<0.05$ ) on days 14 and 21 compared to the No Nitrite control, and on day 19 compared to VJP.

**Table 2.** Least squares means by treatment of log CFU/g *L. monocytogenes* after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g, and with no-HHP treatment.

	Day 0	Day 5	Day 7	Day 12	Day 14	Day 19	Day 21
No Nitrite Control	3.44 <sup>a</sup>	4.14 <sup>a</sup>	4.59 <sup>a</sup>	6.35 <sup>abc</sup>	7.21 <sup>bc</sup>	8.41 <sup>ab</sup>	8.83 <sup>b</sup>
Veg. Pwdr Control	3.35 <sup>a</sup>	3.98 <sup>a</sup>	4.64 <sup>a</sup>	6.47 <sup>bc</sup>	7.28 <sup>bc</sup>	8.50 <sup>b</sup>	8.56 <sup>ab</sup>
Na NO <sub>2</sub> 100 ppm	3.46 <sup>a</sup>	4.00 <sup>a</sup>	4.44 <sup>a</sup>	5.76 <sup>ab</sup>	6.42 <sup>ab</sup>	7.53 <sup>a</sup>	7.93 <sup>ab</sup>
Na NO <sub>2</sub> 200 ppm	3.48 <sup>a</sup>	3.75 <sup>a</sup>	4.14 <sup>a</sup>	5.37 <sup>a</sup>	5.98 <sup>a</sup>	7.49 <sup>a</sup>	7.61 <sup>a</sup>
50 ppm Natural	3.57 <sup>a</sup>	4.21 <sup>a</sup>	4.61 <sup>a</sup>	6.73 <sup>c</sup>	7.44 <sup>c</sup>	8.19 <sup>ab</sup>	8.50 <sup>ab</sup>
100 ppm Natural	3.54 <sup>a</sup>	4.22 <sup>a</sup>	4.74 <sup>a</sup>	6.76 <sup>c</sup>	7.38 <sup>c</sup>	8.26 <sup>ab</sup>	8.45 <sup>ab</sup>
150 ppm Natural	3.52 <sup>a</sup>	3.99 <sup>a</sup>	4.47 <sup>a</sup>	6.40 <sup>bc</sup>	7.00 <sup>bc</sup>	8.05 <sup>ab</sup>	8.27 <sup>ab</sup>
200 ppm Natural	3.29 <sup>a</sup>	3.78 <sup>a</sup>	4.42 <sup>a</sup>	6.04 <sup>abc</sup>	6.69 <sup>abc</sup>	7.95 <sup>ab</sup>	8.26 <sup>ab</sup>
Std. Error	0.11	0.11	0.11	0.11	0.11	0.11	0.11

<sup>a,b,c</sup> Means with different superscripts show differences among means within a column ( $p<0.05$ )

With no-HHP, the No Nitrite control was not significantly different in *L. monocytogenes* growth ( $p>0.05$ ) than all other treatments until day 14 of the sampling



period and then was significantly greater in *L. monocytogenes* growth ( $p < 0.05$ ) than the conventionally cured product (NaNO<sub>2</sub> 200) at days 14 and 21, and trended greater ( $p < 0.10$ ) on day 19. In a previous test (Myers, Montoya, Cannon, Dickson, & Sebranek, 2012) ham products not subjected to HHP and without sodium nitrite showed a greater level of *L. monocytogenes* growth than hams made with sodium nitrite from days 12 through 21 after inoculation. Other researchers have shown that sodium nitrite retards growth of *L. monocytogenes* (Grau & Vanderlinde, 1992; Duffy et al., 1994; Buchanan et al., 1989; Buchanan & Phillips, 1990; McClure et al., 1991), but is not listericidal. Xi et al. (2012) showed that naturally cured hot dogs with 48 ppm nitrite had the same growth rate of *L. monocytogenes* as hot dogs with no added nitrite or nitrate.

**Table 3.** Log<sub>10</sub> reduction in plate count of *L. monocytogenes* after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of 10<sup>5</sup> CFU/g and treatment with either 400 MPa or 600 MPa of high hydrostatic pressure for 3 minutes. (S.E. = 0.19)

Treatments	400 MPa HHP Log <sub>10</sub> Reduction (CFU/g)	600 MPa HHP Log <sub>10</sub> Reduction (CFU/g)
No Nitrite Control	0.82 <sup>ab</sup>	3.84 <sup>a</sup>
Veg. Pwdr Control	0.98 <sup>b</sup>	3.92 <sup>a</sup>
Na NO <sub>2</sub> 100 ppm	0.70 <sup>ab</sup>	3.92 <sup>a</sup>
Na NO <sub>2</sub> 200 ppm	0.59 <sup>a</sup>	3.57 <sup>a</sup>
50 ppm Natural	0.74 <sup>ab</sup>	4.08 <sup>a</sup>
100 ppm Natural	0.72 <sup>ab</sup>	4.25 <sup>a</sup>
150 ppm Natural	0.61 <sup>a</sup>	4.14 <sup>a</sup>
200 ppm Natural	0.62 <sup>a</sup>	3.99 <sup>a</sup>

<sup>a</sup> Means with the same superscript show no differences among means in a column ( $p > 0.05$ )

The reduction in numbers of *L. monocytogenes* after a 5 log CFU/g inoculation and subsequent treatment with HHP are shown in Table 3. Treatment with 600 MPa of

HHP, as expected, gave significantly greater reduction of *L. monocytogenes* numbers than did 400 MPa ( $p < 0.05$ ). There were no significant differences in the numbers of *L. monocytogenes* between treatments within 600 MPa treated samples ( $p > 0.05$ ). For the treatments using 400 MPa pressure, the VJP treatment had significantly greater reduction in *L. monocytogenes* numbers than the NaNO<sub>2</sub>200, 150 Natural, and 200 Natural treatments. The reduction of  $< 1$  log CFU/g of *L. monocytogenes* via 400 MPa treatment would not meet the USDA FSIS definition for an Alternate 2 post-lethality treatment of  $\geq 1$  log reduction of *L. monocytogenes* (FSIS, 2009).

**Table 4.** Least squares means by treatment of log CFU/g *L. monocytogenes* after inoculation with 5-strain mixed culture of *L. monocytogenes* at  $10^3$  CFU/g followed by HHP treatment at 400 MPa for 3 minutes

	Pre-HP	Day 0	Day 14	Day 28	Day 42	Day 56	Day 91
No Nitrite Control	3.14 <sup>a</sup>	2.54 <sup>a</sup>	3.17 <sup>c</sup>	6.18 <sup>b</sup>	7.89 <sup>bc</sup>	8.39 <sup>b</sup>	8.52 <sup>a</sup>
Veg. Pwdr Control	3.04 <sup>a</sup>	2.74 <sup>a</sup>	1.71 <sup>a</sup>	5.67 <sup>b</sup>	8.04 <sup>bc</sup>	8.89 <sup>b</sup>	8.55 <sup>a</sup>
Na NO <sub>2</sub> 100 ppm	3.46 <sup>a</sup>	2.71 <sup>a</sup>	2.09 <sup>ab</sup>	4.32 <sup>a</sup>	7.28 <sup>ab</sup>	8.32 <sup>b</sup>	8.75 <sup>a</sup>
Na NO <sub>2</sub> 200 ppm	3.18 <sup>a</sup>	2.24 <sup>a</sup>	2.26 <sup>abc</sup>	3.88 <sup>a</sup>	6.00 <sup>a</sup>	6.96 <sup>a</sup>	7.60 <sup>a</sup>
50 ppm Natural	3.57 <sup>a</sup>	2.95 <sup>a</sup>	2.46 <sup>abc</sup>	5.75 <sup>b</sup>	8.54 <sup>c</sup>	8.74 <sup>b</sup>	8.49 <sup>a</sup>
100 ppm Natural	3.54 <sup>a</sup>	3.06 <sup>a</sup>	3.05 <sup>bc</sup>	5.95 <sup>b</sup>	8.72 <sup>c</sup>	8.82 <sup>b</sup>	8.70 <sup>a</sup>
150 ppm Natural	3.52 <sup>a</sup>	3.00 <sup>a</sup>	2.65 <sup>abc</sup>	5.40 <sup>b</sup>	8.79 <sup>c</sup>	8.82 <sup>b</sup>	8.65 <sup>a</sup>
200 ppm Natural	3.29 <sup>a</sup>	3.11 <sup>a</sup>	2.58 <sup>abc</sup>	5.93 <sup>b</sup>	8.87 <sup>c</sup>	8.86 <sup>b</sup>	8.76 <sup>a</sup>
Std. Error	0.11	0.12	0.14	0.12	0.14	0.12	0.16

<sup>a,b,c</sup> Means with different superscripts show differences among means within a column ( $p < 0.05$ )

The mean values for *L. monocytogenes* (log CFU/g) of treatments subjected to the lesser pressure level of 400 MPa HHP are shown in Table 4. The treatments were not significantly different on day 0 ( $p > 0.05$ ), but differences in numbers of *L. monocytogenes*

were apparent by day 14. On day 14, the VJP treatment had significantly less *L. monocytogenes* ( $p<0.05$ ) than the No Nitrite and 100 Natural treatments, and less than the initial inoculum. Also, the  $\text{NaNO}_2$ 100 treatment had significantly less *L. monocytogenes* ( $p<0.05$ ) than the No Nitrite treatment at day 14. At days 28, 42, and 56 there were significantly less *L. monocytogenes* ( $p<0.05$ ) for  $\text{NaNO}_2$ 200 compared to all other treatments except the  $\text{NaNO}_2$ 100 treatment on days 28 and 42. The  $\text{NaNO}_2$ 100 treatment also had significantly less *L. monocytogenes* ( $p<0.05$ ) at day 28 compared with all other treatments except  $\text{NaNO}_2$ 200. On day 42, the  $\text{NaNO}_2$ 100 treatment had significantly less *L. monocytogenes* ( $p<0.05$ ) compared to all of the natural treatments (50, 100, 150, and 200 Natural). All samples were not significantly different ( $p>0.05$ ) at 91 days.

**Table 5.** Least squares means by treatment of log CFU/g *L. monocytogenes* after inoculation with a 5-strain mixed culture of *L. monocytogenes* at  $10^3$  CFU/g followed by HHP treatment at 600 MPa for 3 minutes

	Pre-HP	Day 0	Day 28	Day 56	Day 91	Day 119	Day 154	Day 182
No Nitrite	3.14 <sup>a</sup>	0.01 <sup>a</sup>	-0.12 <sup>a</sup>	3.06 <sup>b</sup>	2.28 <sup>ab</sup>	3.71 <sup>b</sup>	3.43 <sup>cd</sup>	2.92 <sup>b</sup>
Veg. JP	3.04 <sup>a</sup>	-0.64 <sup>a</sup>	-0.05 <sup>a</sup>	-0.45 <sup>a</sup>	0.08 <sup>a</sup>	-0.23 <sup>a</sup>	1.35 <sup>abc</sup>	-0.46 <sup>a</sup>
Na NO <sub>2</sub> 100	3.46 <sup>a</sup>	0.38 <sup>a</sup>	-0.01 <sup>a</sup>	0.08 <sup>a</sup>	0.66 <sup>a</sup>	0.16 <sup>a</sup>	2.02 <sup>bc</sup>	1.40 <sup>ab</sup>
Na NO <sub>2</sub> 200	3.18 <sup>a</sup>	-0.22 <sup>a</sup>	-0.92 <sup>a</sup>	-0.92 <sup>a</sup>	0.57 <sup>a</sup>	-0.23 <sup>a</sup>	0.08 <sup>ab</sup>	-0.92 <sup>a</sup>
50 Natural	3.57 <sup>a</sup>	-0.08 <sup>a</sup>	-0.39 <sup>a</sup>	0.41 <sup>a</sup>	0.23 <sup>a</sup>	0.03 <sup>a</sup>	-0.39 <sup>a</sup>	-0.39 <sup>a</sup>
100 Natural	3.54 <sup>a</sup>	0.38 <sup>a</sup>	0.33 <sup>a</sup>	0.97 <sup>ab</sup>	1.06 <sup>a</sup>	1.84 <sup>ab</sup>	-0.23 <sup>ab</sup>	1.37 <sup>ab</sup>
150 Natural	3.52 <sup>a</sup>	0.85 <sup>a</sup>	0.64 <sup>a</sup>	0.17 <sup>a</sup>	3.74 <sup>bc</sup>	4.34 <sup>bc</sup>	5.62 <sup>de</sup>	5.84 <sup>c</sup>
200 Natural	3.29 <sup>a</sup>	1.00 <sup>a</sup>	0.54 <sup>a</sup>	2.79 <sup>b</sup>	5.17 <sup>c</sup>	8.14 <sup>c</sup>	7.43 <sup>e</sup>	6.96 <sup>c</sup>
Std. Error	0.11	0.33	0.33	0.33	0.33	0.33	0.33	0.33

<sup>a,b,c,d,e</sup> Means with the same superscript show no differences among means in a column ( $p>0.05$ )

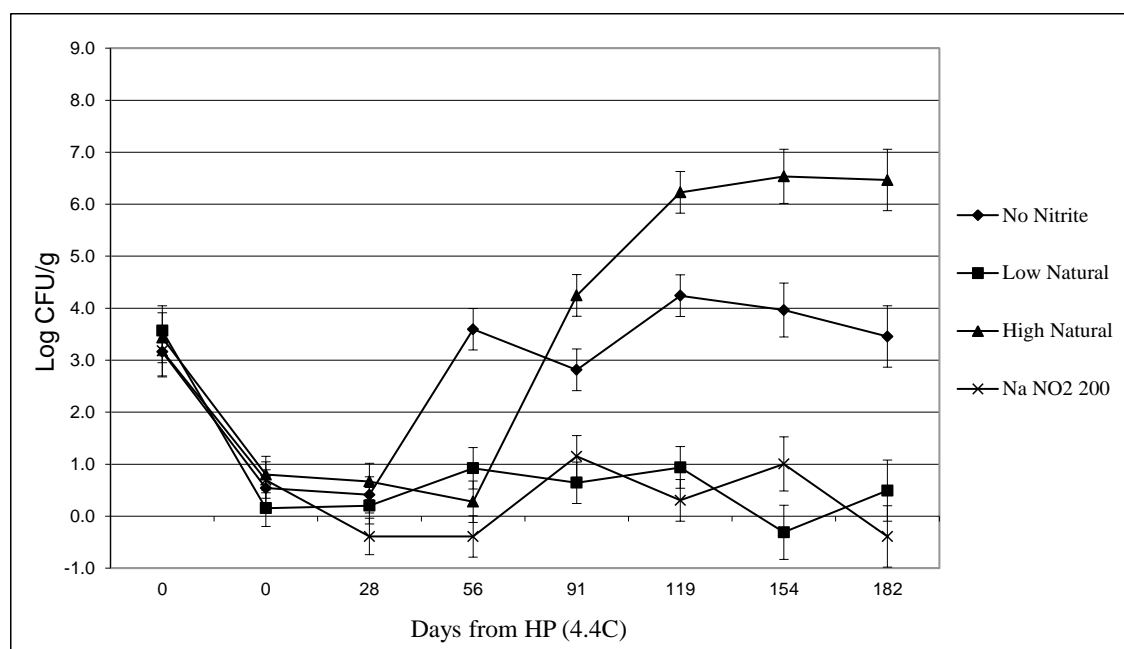
For the samples inoculated at 3 log CFU/g and then subjected to the greater pressure level of 600 MPa HHP for 3 minutes, Table 5 shows the change in number of *L.*

*monocytogenes* over the 182 day evaluation for each treatment. A reduction in *L. monocytogenes* numbers of about 2.3 - 3.0 log CFU/g was achieved immediately after HHP and mean values for all treatments were at or below the detection limit of 1.0 log CFU/g. There were no significant treatment differences immediately after pressure treatment or after 28 days ( $p>0.05$ ). At day 56, the No Nitrite and 200 Natural treatments were significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers than all treatments, other than the 100 Natural treatment. At days 119 through 182, the 200 Natural treatment was significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers than all other treatments except the 150 Natural. The No Nitrite treatment was not significantly different ( $p>0.05$ ) from 150 Natural on days 91-154, but had significantly less *L. monocytogenes* ( $p<0.05$ ) on day 182. The No Nitrite treatment was significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers than NaNO<sub>2</sub>200 and 50 Natural treatments on days 119 and 182, and significantly greater ( $p<0.05$ ) than NaNO<sub>2</sub>100 on day 119.

**Table 6.** Counts of *L. monocytogenes* exceeding  $10^2$  at 154 and 182 days of shelf life after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g followed by HHP treatment at 600 MPa for 3 minutes (two or three replications with three measurements at each replication).

<b>Treatments:</b>	<b>Day 154</b> Counts > $10^2$ CFU/g	<b>Day 182</b> Counts > $10^2$ CFU/g
No Nitrite Control	3 of 6	3 of 6
Veg. Pwdr Control	1 of 6	0 of 6
Na NO <sub>2</sub> 100 ppm	3 of 9	2 of 9
Na NO <sub>2</sub> 200 ppm	0 of 6	0 of 6
50 ppm Natural	0 of 9	0 of 9
100 ppm Natural	0 of 9	2 of 9
150 ppm Natural	7 of 9	7 of 9
200 ppm Natural	8 of 9	8 of 9

Table 6 shows further detail on the number of samples for each treatment that exhibited growth of  $>2.0$  log CFU/g either at 154 or 182 days. The 150 Natural and 200 Natural treatments had seven and eight of nine total measures, respectively that showed growth on these days. The 50 Natural and NaNO<sub>2</sub>200 treatments had no growth in all measurements at both 154 and 182 days.



**Fig. 1.** Average log<sub>10</sub> CFU/g of no nitrite, low natural nitrite (50 & 100 ppm targets), high natural nitrite (150 & 200 ppm targets), and 200 ppm sodium nitrite concentrations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of 10<sup>3</sup> CFU/g followed by HHP treatment at 600 MPa for 3 minutes (Detection limit=1.0 Log<sub>10</sub> CFU/g).

As a way of further evaluating the results of the 3 log CFU/g inoculation and treatment with 600 MPa HHP, Figure 1 shows the average means of low natural nitrite (50 and 100 Natural treatments), high natural nitrite (150 and 200 Natural treatments), no nitrite, and NaNO<sub>2</sub>200 treatments. Sindelar et al. (2007b) used treatments of about 69 and 120 ppm formulated nitrate via vegetable juice powder to achieve concentrations of

19.5 and 36.1 ppm, respectively, of residual nitrite after incubation, prior to cooking. Jackson, Sullivan, Kulchaiyawat, Sebranek, and Dickson (2011) showed that natural commercial hams had residual nitrite concentrations of ca. 5-13 ppm. Data from these two studies compare to similar concentrations of residual nitrite after cooking/HHP for the 50 Natural and 100 Natural treatments, as shown in Table 7 and Figure 2. Therefore, the low average level of about 75 ppm formulated nitrite shown in Figure 1 is similar to these concentrations and had *L. monocytogenes* numbers that were not significantly different than the conventional nitrite concentrations of the NaNO<sub>2</sub>200 treatment.

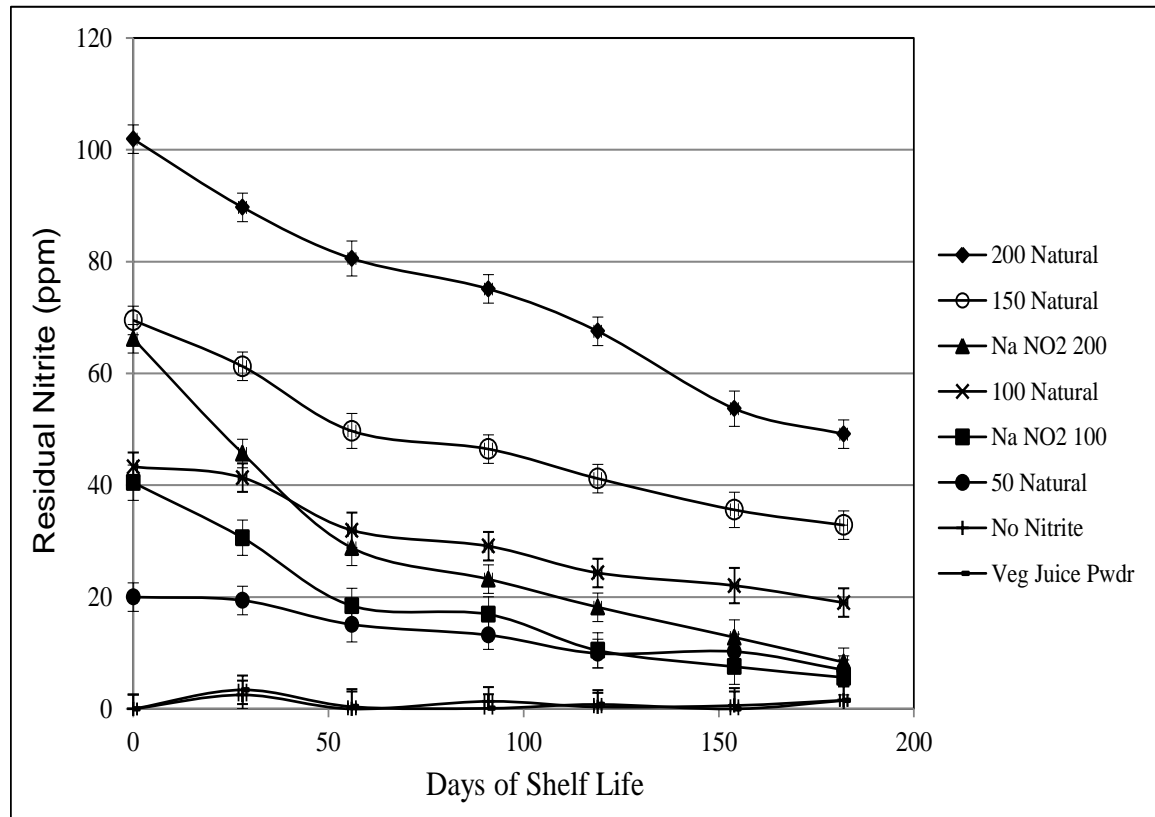
**Table 7.** Average residual nitrite values (ppm) for treatments with added sodium nitrite or added nitrite-containing vegetable juice powder, before and after HHP treatment at 600 MPa for 10 minutes.

Treatments	Before HHP	After HHP
Na NO <sub>2</sub> 100 ppm	42.9 <sup>b</sup> ±9.7	40.5 <sup>b</sup> ±9.3
Na NO <sub>2</sub> 200 ppm	67.0 <sup>c</sup> ±2.1	66.2 <sup>c</sup> ±5.5
50 ppm Natural	22.6 <sup>a</sup> ±3.2	20.0 <sup>a</sup> ±3.5
100 ppm Natural	44.5 <sup>b</sup> ±7.7	43.3 <sup>b</sup> ±4.3
150 ppm Natural	71.0 <sup>c</sup> ±5.9	69.5 <sup>c</sup> ±4.7
200 ppm Natural	103.8 <sup>d</sup> ±7.5	101.9 <sup>d</sup> ±5.3

<sup>a,b,c,d</sup> Means with different superscripts show differences among LS means in columns (p<0.05)

Residual nitrite values were evaluated before and after 10 minute HHP treatment (600 MPa) for the samples containing nitrite (Table 7). The average least squares mean for residual nitrite was 1.8 ppm less after HHP treatment, but this was not a significant difference (p>0.05). Residual nitrite levels of the HHP samples over the testing period are shown in Figure 2. The greatest residual nitrite concentration after cooking, slicing,

and HHP was the 200 Natural treatment, which was significantly greater ( $p<0.05$ ) than all other treatments at 108 ppm. Levels of residual nitrite for all treatments decreased over the time period of the study.



**Fig. 2.** Average residual nitrite level of formulations over shelf life (uninoculated samples with 10 minute HHP treatment).

Other researchers have observed gradual decreases in residual nitrite during cured meat storage (Jantawat, Runglerdkriangkrai, Thunpithayakul, & Sanguandeeikul, 1993; Hustad et al. 1973; Sindelar et al., 2007b). The 200 Natural treatment had significantly greater concentrations of residual nitrite ( $p<0.05$ ) throughout the storage period, compared to all other treatments. The 150 Natural treatment had significantly lesser concentrations of residual nitrite ( $p<0.05$ ) throughout the study compared to the 200 Natural treatment, but significantly greater concentrations than all other treatments

( $p < 0.05$ ) except for the  $\text{NaNO}_2$ 200 treatment on day 0. The control treatments with No Nitrite and VJP both had significantly less residual nitrite than all other treatments ( $p < 0.05$ ) throughout the length of the study. Duffy et al. (1994) in fact showed that increased concentrations of residual nitrite gave increased inhibition of *L. monocytogenes* in vacuum-packaged ready-to-eat meats produced with added sodium nitrite. Therefore the results of the *L. monocytogenes* inoculation studies with increased growth at the greater concentrations of ingoing and residual nitrite do not match findings from previous studies.

It is not clear why the greater concentrations (150 and 200 ppm) of pre-converted vegetable juice powder had a greater growth rate for *L. monocytogenes* than the lesser concentrations (50 and 100 ppm nitrite). Since the vegetable juice powder used as the natural nitrite source contained just 2.25% nitrite, it must be added at a relatively greater concentration (0.67-0.89%) to achieve the 150-200 ppm nitrite concentrations. This means that there is 97.75% of the ingredient that could potentially provide some level of unknown beneficial nutrients such as vitamins, minerals or other growth factors to prompt *L. monocytogenes* growth, and thus when used at the greater levels of addition in the 150 and 200 ppm natural formulations could potentially provide conditions that give increased growth of *L. monocytogenes* as suggested by these results.

The LS means for moisture, fat, protein, salt, and pH by treatment are shown in Table 8. There were no significant differences between treatments in moisture, fat, or protein values ( $p > 0.05$ ). However, the least squares mean for pH for 200 Natural at 6.63 was significantly greater ( $p < 0.05$ ) than all other treatments and pH for 150 Natural at 6.48 was significantly greater ( $p < 0.05$ ) than all treatments except 100 Natural (6.39) and



NaNO<sub>2</sub>100 (6.36) treatments, and was numerically greater than all treatments except 200 Natural. Borges, Silva, and Teixeira (2011) found that increased pH levels from 4.2 to 6.5 increased the growth rate of *L. monocytogenes*. Vermeulen et al. (2007) also found that *L. monocytogenes* exhibited increased growth within a greater pH range in their test of multiple factors influencing *L. monocytogenes* growth. This suggests that the increased growth rate of *L. monocytogenes* in the 150 and 200 Natural formulations could be due to the increased pH level in these items.

**Table 8.** Least squares means for percent finished product moisture, fat, protein, and salt, as well as finished product pH.

	<b>Moisture</b>	<b>Fat</b>	<b>Protein</b>	<b>Salt</b>	<b>pH</b>
No Nitrite Control	76.29 <sup>a</sup>	1.88 <sup>a</sup>	17.94 <sup>a</sup>	2.47 <sup>ab</sup>	6.12 <sup>a</sup>
Veg. Pwdr Control	75.93 <sup>a</sup>	1.74 <sup>a</sup>	18.19 <sup>a</sup>	2.42 <sup>a</sup>	6.14 <sup>ab</sup>
Na NO <sub>2</sub> 100 ppm	75.88 <sup>a</sup>	1.38 <sup>a</sup>	18.38 <sup>a</sup>	2.40 <sup>a</sup>	6.36 <sup>cd</sup>
Na NO <sub>2</sub> 200 ppm	75.70 <sup>a</sup>	1.61 <sup>a</sup>	18.38 <sup>a</sup>	2.41 <sup>a</sup>	6.32 <sup>c</sup>
50 ppm Natural	76.34 <sup>a</sup>	1.69 <sup>a</sup>	18.07 <sup>a</sup>	2.44 <sup>ab</sup>	6.28 <sup>bc</sup>
100 ppm Natural	75.96 <sup>a</sup>	1.54 <sup>a</sup>	18.30 <sup>a</sup>	2.51 <sup>bc</sup>	6.39 <sup>cd</sup>
150 ppm Natural	76.05 <sup>a</sup>	1.64 <sup>a</sup>	18.04 <sup>a</sup>	2.58 <sup>c</sup>	6.48 <sup>d</sup>
200 ppm Natural	75.72 <sup>a</sup>	1.70 <sup>a</sup>	17.91 <sup>a</sup>	2.68 <sup>d</sup>	6.63 <sup>e</sup>
Std. Error	0.26	0.24	0.28	0.04	0.07

<sup>a,b,c,d,e</sup> Means with different superscripts show differences among LS means in columns (p<0.05)

The salt level of the 200 Natural treatment was also significantly greater (p<0.05) than all other treatments. Researchers have found that greater levels of salt (sodium chloride) gives a slight negative effect on *L. monocytogenes* growth (Glass & Doyle

1989; McClure et al. 1991; Seman, Borger, Meyer, Hall, & Milkowski, 2002; Legan, Seman, Milkowski, Hirschey, & Vandeven, 2004), but the effect was very small so it seems unlikely that salt concentration influenced growth of *L. monocytogenes* in this study.

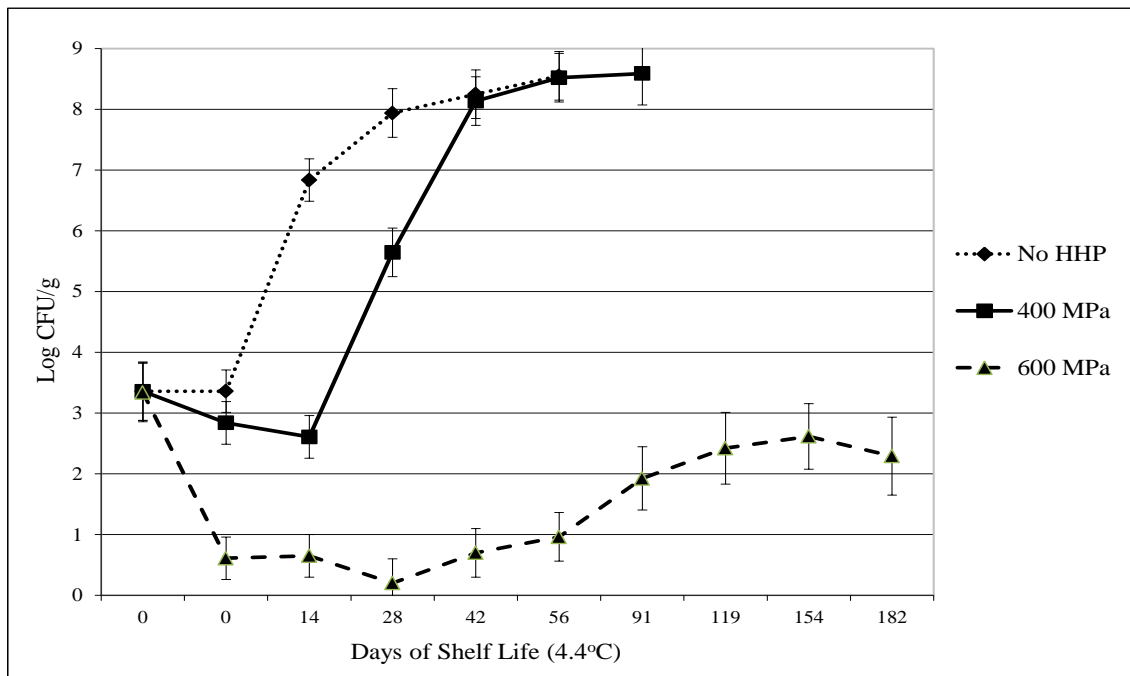
**Table 9.** Least squares mean of residual nitrate concentrations (ppm) over the duration of the study.

	Day 0	Day 28	Day 56	Day 91	Day 119	Day 154	Day 182
No Nitrite	0.45 <sup>c</sup>	0.00 <sup>d</sup>	1.50 <sup>d</sup>	0.20 <sup>d</sup>	3.25 <sup>d</sup>	0.40 <sup>d</sup>	0.85 <sup>d</sup>
Veg. Juice Pwdr.	79.00 <sup>a</sup>	67.80 <sup>a</sup>	73.70 <sup>a</sup>	66.55 <sup>a</sup>	83.65 <sup>a</sup>	82.55 <sup>a</sup>	76.85 <sup>a</sup>
Na NO <sub>2</sub> 100 ppm	13.50 <sup>b</sup>	5.15 <sup>bc</sup>	7.05 <sup>cd</sup>	11.43 <sup>bc</sup>	17.70 <sup>c</sup>	20.80 <sup>bc</sup>	22.67 <sup>b</sup>
Na NO <sub>2</sub> 200 ppm	8.75 <sup>bc</sup>	6.95 <sup>bc</sup>	7.80 <sup>cd</sup>	6.40 <sup>bcd</sup>	15.75 <sup>c</sup>	18.40 <sup>c</sup>	15.10 <sup>bc</sup>
50 ppm Natural	11.45 <sup>b</sup>	4.60 <sup>bc</sup>	6.55 <sup>cd</sup>	5.07 <sup>cd</sup>	15.83 <sup>c</sup>	16.25 <sup>c</sup>	11.93 <sup>c</sup>
100 ppm Natural	14.30 <sup>b</sup>	6.25 <sup>bc</sup>	9.25 <sup>cd</sup>	9.63 <sup>cd</sup>	18.87 <sup>c</sup>	18.80 <sup>c</sup>	16.90 <sup>bc</sup>
150 ppm Natural	16.30 <sup>b</sup>	10.80 <sup>b</sup>	15.40 <sup>bc</sup>	15.53 <sup>bc</sup>	20.73 <sup>bc</sup>	31.55 <sup>b</sup>	18.97 <sup>bc</sup>
200 ppm Natural	18.50 <sup>b</sup>	19.30 <sup>b</sup>	25.00 <sup>b</sup>	20.27 <sup>b</sup>	24.47 <sup>b</sup>	21.75 <sup>bc</sup>	22.40 <sup>b</sup>
Avg. LS Mean of 6 nitrite trtmts.	13.80	8.84	11.84	11.39	18.89	18.29	17.98
Std. Error	1.67	2.17	7.21	5.69	4.92	2.91	4.89

<sup>a,b,c,d</sup> Means with different superscripts show differences among LS means in columns ( $p < 0.05$ )

The least squares means for residual nitrate are shown in Table 9. The level of residual nitrate in the non-converted VJP stayed at a relatively constant level over the shelf life of product and was significantly greater ( $p < 0.05$ ) than all other treatments on all days, with the exception of NaNO<sub>2</sub>200 on day 0. Since the VJP formulation did not contain any nitrate-reducing starter culture and the cook process did not include a fermentation step, the nitrate could not be converted to nitrite during the cooking process,

as shown also by low residual nitrite concentrations in Figure 2, and thus remained as nitrate in the cooked ham. The average level of residual nitrate for natural and conventional treatments that contained added sodium nitrite decreased slightly at day 28, increased slightly at days 56 and 91, then remained relatively steady throughout the remaining sampling period (Table 9). Other researchers have observed residual nitrate levels increasing over product shelf life (Cassens, Greaser, & Lee, 1979; Terns, Milkowski, Rankin, & Sindelar, 2011). Fujimaki, Emi, and Okitani (1975) showed that as nitrite is converted to nitric oxide, a portion is also converted to nitrate. In a study of cured bacon Herring (1973) also found that residual nitrate increased over the shelf life of product and attributed this increase in part to the oxidation of nitric oxide or nitrous oxide with nitrate being one product of the reaction.



**Fig. 3.** Least Squares Means of *L. monocytogenes* CFU/g by HHP treatment after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g followed by no HHP, 400 MPa HHP treatment for 3 minutes, and 600 MPa HHP treatment for 3 minutes (detection limit =  $1.0 \log_{10}$  CFU/g).

The effect of HHP treatment on *L. monocytogenes* growth is shown in Figure 3. The use of 400 MPa HHP provided only a slight reduction of <1.0 log CFU/g of *L. monocytogenes* immediately after treatment, but the numbers increased rapidly and were similar to no-HHP product after 42 days. Treatment with 600 MPa resulted in a significant decrease ( $p < 0.05$ ) of about 3 log CFU/g and inhibited growth to high levels throughout shelf life, although there were some treatment differences as shown previously in Tables 4 and 5.

**Table 10.** Least square mean of Minolta colorimeter L\*, a\*, and b\* values.

	L*	a*	b*
No Nitrite Control	62.80 <sup>c</sup> ±1.35	7.82 <sup>a</sup> ±1.44	5.87 <sup>d</sup> ±0.83
Veg. Pwdr Control	63.02 <sup>c</sup> ±1.11	7.80 <sup>a</sup> ±1.49	5.89 <sup>d</sup> ±0.82
Na NO <sub>2</sub> 100 ppm	58.94 <sup>ab</sup> ±2.02	12.63 <sup>bc</sup> ±1.04	2.38 <sup>a</sup> ±0.25
Na NO <sub>2</sub> 200 ppm	59.87 <sup>b</sup> ±2.85	12.87 <sup>c</sup> ±1.09	2.55 <sup>a</sup> ±0.30
50 ppm Natural	58.89 <sup>ab</sup> ±0.92	12.88 <sup>c</sup> ±0.55	3.38 <sup>b</sup> ±0.19
100 ppm Natural	58.60 <sup>ab</sup> ±0.35	12.50 <sup>bc</sup> ±0.32	3.48 <sup>bc</sup> ±0.04
150 ppm Natural	58.25 <sup>ab</sup> ±0.71	12.43 <sup>bc</sup> ±0.28	3.76 <sup>bc</sup> ±0.12
200 ppm Natural	57.74 <sup>a</sup> ±0.40	11.92 <sup>b</sup> ±0.44	3.95 <sup>c</sup> ±0.24

<sup>a,b,c,d</sup> Means with different superscripts show differences among LS means in columns ( $p < 0.05$ )

Results of the Minolta colorimeter analyses are shown in Table 10. As expected, the overall color of the samples without nitrite (No Nitrite and VJP) was lighter, less red, and more yellow than the remaining treatments that contained nitrite. The No Nitrite and VJP treatments were significantly lighter ( $p < 0.05$ ) in color (greater in L\* value) than all other treatments, with the NaNO<sub>2</sub>200 treatment being significantly lighter than the 200 Natural treatment. For the redness (a\*) value, the No Nitrite and VJP treatments were

significantly less red ( $p < 0.05$ ) than all other treatments and the NaNO<sub>2</sub>200 and 50 Natural treatments were significantly darker than the 200 Natural treatment. For the yellowness ( $b^*$ ) value, the No Nitrite and VJP treatments were significantly more yellow than all other treatments. The NaNO<sub>2</sub>100 and NaNO<sub>2</sub>200 treatments had a  $b^*$  value that was less than all other treatments with the 50 Natural treatment also having a reduced  $b^*$  value compared to the 200 Natural treatment.

#### 4. Conclusions

This study showed that there were significant differences in the growth rate of *L. monocytogenes* due to HHP treatments and both the type and concentration of nitrite in the formulation. The use of HHP at 600 MPa reduced numbers of *L. monocytogenes* by 2-3 log CFU/g when inoculated at 10<sup>3</sup> CFU/g and >3.5 log CFU/g when inoculated at 10<sup>5</sup> CFU/g. However, the use of 400 MPa for 3 minutes was not sufficient to reduce numbers of *L. monocytogenes* by more than one log CFU/g and the organism was able to quickly repair and grow to high levels when inoculated at 10<sup>3</sup> CFU/g.

Use of sodium nitrite at 200 ppm inhibited growth of *L. monocytogenes* more than use of natural nitrite at 150 or 200 ppm of formulated nitrite. Use of 50 or 100 ppm nitrite from a natural source was not significantly different than the sodium nitrite-cured products. However, use of 150 or 200 ppm natural nitrite resulted in significantly greater numbers of *L. monocytogenes* from day 56 to the end of the evaluation period of 182 days. It is possible that an increased pH in the naturally-cured products may have contributed to an increased level of growth or it is possible that other nutrients within the vegetable juice powder, when added at the high concentrations may have offset the inhibitory effects of nitrite and resulted in increased growth of *L. monocytogenes*.

Further research is needed to understand the reason for the greater level of growth in items with increased ingoing and residual nitrite from the vegetable juice powder, as other researchers have found the opposite to be the case for the addition of the chemical form of sodium nitrite (Buchanan et al., 1989; Grau & Vanderlinde 1992; Duffy et al., 1994; Farber & Daley, 1994). There is also the possibility that the inoculum level of 3 log CFU/g is so close to the maximum level of eradication of *L. monocytogenes* by HHP that growth observed was random in nature and not due to treatment differences.

Other researchers have pointed out the benefit in the use of a reducing agent along with nitrite to provide increased inhibition of *L. monocytogenes* in ready-to-eat meats (Sebranek & Bacus, 2007; Xi et al., 2012). Some researchers have tested use of cherry powder as a reducing agent in natural meat items (Terns, Milkowski, Rankin, & Sindelar, 2011). Further research regarding the effects of cherry juice powder or other reducing agents in conjunction with natural sources of nitrite on inhibition of *L. monocytogenes* in ready-to-eat natural meats is also needed.

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## **CHAPTER 4 - Effects of High Hydrostatic Pressure, Antimicrobial Ingredients, and Antimicrobial Sprays on Growth of *Listeria monocytogenes* in Conventional and Natural Formulations of RTE Sliced Ham**

A paper to be submitted to the *Journal of Food Protection*

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### **ABSTRACT**

Growth of *Listeria monocytogenes* in ready-to-eat (RTE) ham was evaluated in both conventional (sodium nitrite added) and natural (nitrite from vegetable juice source) formulations. Control treatments of both formulation types, which used no antimicrobial ingredients, were compared to treatments with antimicrobial ingredients added to the raw meat mixture and also compared to treatments with antimicrobial sprays added to the sliced RTE product. High hydrostatic pressure (HHP) processing was also carried out on each treatment for 0, 3, and 6 minutes at 400 MPa. *L. monocytogenes* grew rapidly to high numbers ( $<10^8$  CFU/g) in conventional and natural control treatments within a few weeks, regardless of HHP time. Addition of antimicrobial ingredients or ingredient/spray combinations in natural formulas maintained *L. monocytogenes* at less than the inoculum level throughout the 154-day study, with or without the use of HHP. Conventional formulations with antimicrobial ingredients or ingredient/spray combinations without HHP did not restrict *L. monocytogenes* growth, as *L. monocytogenes* was above the inoculum level after 28 days in all conventional treatments. However the addition of ingredients or ingredient/spray combination with HHP in conventional formulations maintained *L. monocytogenes* at below inoculum levels throughout the 154-day study.

## INTRODUCTION

*L. monocytogenes* has the capability of growing at refrigerated temperatures, making it a pathogen of high concern for ready-to-eat (RTE) meat items that are exposed to the environment after the lethality cook step (33, 47). Among foodborne pathogens, *L. monocytogenes* has among the greatest hospitalization rates and mortality rates (84). A study of foodborne outbreaks in the United States between 1998 and 2002 found that *L. monocytogenes* caused 54% of all deaths (54). Commercial cooking processes for meats are designed to provide an adequate kill of *L. monocytogenes* during lethality cooking of finished products (48, 83), but contamination with *L. monocytogenes* can potentially occur after the lethality cook step via personnel or equipment transferring contamination to the ready-to-eat product (52, 65, 97). If unimpeded, this contamination can result in growth of *L. monocytogenes*, which, depending on virulence of the specific strain, the immune condition of the consumer, and other potential factors, could potentially result in infection, illness, and even death (43, 47, 61, 76, 95).

A number of studies have found that traditional ingredients used in ready-to-eat meat items such as salt (39, 50, 60, 86) and sodium nitrite (15, 29, 41, 60, 85) can provide some level of *L. monocytogenes* inhibition, but are not listericidal. A number of antimicrobials have also been researched and several have been found to provide some level of *L. monocytogenes* control in ready-to-eat meats including sodium/potassium lactate (6, 55, 59, 62, 74, 92), combinations of lactate with diacetate (44, 50, 86), octanoic acid (16), and lauric arginate (LAE) (53, 57, 75, 89). Other studies have pointed to the mechanism of effect for most antimicrobials as undissociated acid molecules entering a bacterial or fungal cell and then becoming dissociated. The release of protons causes

disruption of the proton-motive force of the organism, which can lead to cell damage or death due to large amount of the stored adenosine triphosphate (ATP) being expended in pumping protons out of the cell in an attempt to maintain pH equilibrium (9, 12, 13, 28, 30, 49, 82, 91). Antimicrobials may also act by causing damage to microbial cell membranes, or disruption of the proton-motive force may ultimately cause irreparable damage to the cell membrane (1, 17, 28, 36). However, because antimicrobials are applied during the process of formulating or before final packaging, recontamination with *L. monocytogenes* could occur and allow for survival and growth of the organism (28, 44, 48, 51).

High hydrostatic pressure (HHP) has also been used in RTE meats to provide control of bacterial pathogens (58, 68, 87). HHP-processing of RTE meats is applied after the product is in the final package, so it provides additional assurance that products will not be recontaminated, and HHP applies pressure isostatically so is not dependent on size or shape of the product and package (7, 8, 34, 58, 68, 87). Food processors have used HHP to provide assurance of product safety and consistent quality in natural, organic, and preservative-free products (7, 8, 19, 27, 34, 68, 87). Researchers have shown that HHP, at pressures of  $\geq 600$  MPa, provides a significant reduction of foodborne pathogens in RTE meats (7, 38, 45, 71, 98). The mode of action of HHP on pathogens has been shown to be disruption of cell membranes (18, 68, 77, 78, 87), although the membrane damage ultimately triggers further cellular changes that may be the cause of microbial inactivation or destruction (7, 69).

Unlike inactivation of most pathogens subjected to heat pasteurization, the death rate of organisms subjected to HHP is not always linear. Some studies have found death

rates closely follow first-order kinetics (63, 73), while others have found a non-linear relationship with tailing behavior in some apparently pressure-tolerant survivors (20, 34, 88). This tailing effect may be caused by different tolerances to HHP by various strains or could be due to some level of adaptation to stress (2, 20, 38, 90).

To overcome some of the potential differences in inactivation of HHP, some studies have tested combinations of antimicrobial ingredients and HHP to provide multiple opportunities to weaken the cell membrane of the pathogen and provide an enhanced effect over the use of HHP or antimicrobials alone (6, 23, 46, 56). Other antimicrobial ingredients such as lauric arginate, octanoic acid, vinegar, nisin, and bacteriocins may be effective in microbial control via mechanisms that include weakening of the cell membrane of vegetative organisms (11, 16, 31, 40, 53, 80, 81, 89, 99). Most of these ingredients have not been tested in combination with HHP for potential pathogen control.

The purpose of this study is to examine the use of HHP at a moderate level of pressure (400 MPa), in combination with commercially-available antimicrobial compounds for destruction and continued inhibition of *Listeria monocytogenes* in natural and conventional sliced meats.

## MATERIALS AND METHODS

**Product manufacture.** Fresh trimmed ham muscles from the inside (*semimembranosus*) and knuckle (*rectus femoris*, *vastus intermedius*, *vastus lateralis*, and *vastus medialis*) were used for manufacture of hams. All raw materials were used within 2-4 days after harvest and were ground using a 0.3175 cm diameter plate immediately



before use. After grinding, meats were mixed in a Blentech Auto Chef Silver Ribbon blender (Blentech Corp., Rohnert Park, CA.) for ca. 1 minute to assure homogeneity and randomly assigned to treatments. Formulation information is detailed in Table 1.

TABLE 1. *Ingredient formulations for natural or conventional formulations*

	Conventional Formulations		Natural Formulations	
	Control	Antimicrobial	Control	Antimicrobial
Salt	2.40%	2.40%	2.40%	2.40%
Sugar	1.00%	1.00%	1.00%	1.00%
Ham	80.00%	80.00%	81.97%	81.97%
Water	16.14%	15.28%	14.27%	11.77%
Sodium Phosphate	0.40%	0.40%	-	-
Sodium Nitrite	200 ppm*	200 ppm*	-	-
Sodium Erythorbate	500 ppm*	500 ppm*	-	-
VegStable 506	-	-	.4444%*	.4444%*
MOstatin V	-	-	-	2.50%
LM220	-	0.86%	-	-

\* percentage of total meat weight

All formulations consisted of 1% sucrose (United Sugars, Bloomington, MN.) and 2.4% salt (sodium chloride - Morton Salt, Chicago, IL.). Conventionally cured products contained 0.4% sodium tripolyphosphate (ICL Performance Products LP, St. Louis, MO.), 500 ppm sodium erythorbate, and 200 ppm sodium nitrite, based on raw meat weight. Products without added sodium nitrite, but containing “natural nitrite” were formulated using VegStable 506 (Florida Food Products, Eustis, FL.) at 0.4444%, which at a nitrite concentration of 22,500 ppm gave a concentration of 100 ppm of formulated nitrite based on raw meat weight. A screening test was conducted to determine

ingredients to use in either the natural or conventionally-cured items to provide effects that were additive or synergistic with high hydrostatic pressure. The ingredient chosen for use in the conventionally cured product was a commercially-available blend of cultured dextrose, nisin, rosemary extract, and salt (LM220; Danisco USA, New Century, KS). The ingredient used for the natural product was commercially-available cultured vinegar (MOstatin V; World Technology Ingredients, Jefferson, GA.). Water was adjusted to achieve 22% total added ingredients, based on raw meat weight, in the natural formulations and 25% total added ingredient in the conventional formulations.

Non-meat ingredients were dissolved in 85% of the water using a Lightnin mixer (Model S1UO3A, Lightnin, Rochester, NY) and additional water/ice was added to achieve a temperature of 28°F in the pickle solution. The pickle solution was then added along with the meat materials to the Blentech mixer and blended under vacuum for 20 minutes at 30 rpm. After blending, the mixed batter was stuffed into 8.38 cm diameter, non-permeable casings (Viscofan, Danville, IL) and held for 18 hours at 2°C before cooking. All treatments for each replication were cooked together via steam heat for 45 min. at 54°C, 45 min. at 63°C, 45 min. at 71°C, and ca. 1 hour at 80°C to an internal temperature of 74°C. After reaching final temperature, cooked products were showered with ca. 21°C water for 30 minutes and then chilled in a 1°C cooler to reach an internal temperature of <4°C within 6 hours of cooking.

Within 1 week after cooking, the casings were removed and each product treatment was sliced into 11 gram slices. Slices were stacked and bulk packaged into ~2 pound vacuum packages (Curwood, Inc., Oshkosh, WI). Because several studies have shown that *L. monocytogenes* growth may be affected by increased concentrations of

lactic acid bacteria (14, 32, 35), samples were treated with high pressure at 600 MPa for 10 minutes to greatly decrease the number of vegetative organisms potentially acquired during slicing/packaging.

**Microbiological procedures.** Each *L. monocytogenes* culture was grown overnight (18-24 hours) in tryptic soy broth (TSB) at 35°C and each culture was tested for purity on modified oxford agar (MOX) (VWR, Batavia, IL). After <1 week storage at 2°C, individual 11 gram ham slices were repackaged into 13 by 29 cm packages (oxygen transmission rate = 3.5 cc/100 sq. in./day; Ultravac Solutions, Kansas City, MO.) for inoculation. *Listeria monocytogenes* strains used for the study were ATCC 7644, NCTC 10890, ATCC 19112, ATCC 19114, and ATCC 19115 (Microbiologics, St. Cloud, MN.). Equal amounts of each strain were mixed into a common mixed culture used for the inoculation. Based on prior testing, a count of  $10^9$  cfu/ml of *L. monocytogenes* in the overnight cultures was used for calculation of further dilutions. The mixed culture was diluted using sterile 0.1% peptone water to achieve two targeted levels of inoculum of  $10^5$  and  $10^3$  CFU/gram. Each level of inoculum was added to an 11-gram slice to achieve the targeted level. After inoculation, all samples were vacuum-sealed on a Multivac packaging machine (model A300; Multivac, Kansas City, MO.).

The treatments for both the conventional and natural formulations are shown in Table 2. The inoculated samples for each treatment, at each level of inoculation, were randomly assigned to one of three groups consisting of 1) Non-HHP samples, 2) 400 MPa HHP for 180 seconds, or 3) 400 MPa HHP for 360 seconds. After inoculation, all samples that didn't require spray treatment were vacuum-sealed on a Multivac packaging machine (model A300; Multivac, Kansas City, MO.) within ca. 30 minutes. At ca. 45

minutes after inoculation, an antimicrobial spray was added to designated treatments. A spray bottle with a misting spray nozzle (US Plastics Corp., Lima, OH.) was used to dispense the antimicrobial at a quantity of 0.143 g onto the top surface of the ham slice. For the conventional treatments the spray was dispensed to deliver 200 ppm of lauric arginate (LAE) (Protect-M, Purac America, Inc., Lincolnshire, IL) onto the surface of the slice. For the natural treatments, the spray was dispensed to deliver either 44 ppm of lauric arginate or 400 ppm octanoic acid (OA) (OctaGone, Ecolab, St. Paul, MN). Ingredient quantities were determined by Food Safety and Inspection Service (FSIS) limits as set by FSIS Directive 7120.1 (37). After spraying, all samples were vacuum-sealed on the Multivac packaging machine within ca. 30 minutes of spray application.

TABLE 2. *Treatment used for conventional and natural formulations*

	Conventional Formulations		Natural Formulations	
	Control	MOstatin	Control	LM220
No Spray	X	X	X	X
44 ppm LAE	-	-	-	X
400 ppm OA	-	-	-	X
200 ppm LAE	X	X	-	-

The experiment was replicated three times with microbial analyses completed in triplicate for each replication on each testing date. The samples of all treatments were stored at 4.4°C throughout the duration of the experiment. For the  $10^5$  inoculum level, evaluations were performed only on day 0 to determine *L. monocytogenes* population differences before and after HHP treatment. All treatments at  $10^3$  inoculation were evaluated at days 0, 14, 28, 42, 56, 70, 91, 119, 154, and 182 after inoculation and the

non-HHP samples were also evaluated on days 5, 7, 12, 19, and 21.

For the non-HHP treated group, the samples were inoculated and the day 0 microbial measurements conducted for each treatment at ca. 2 hours after being inoculated. For the HHP-treated samples on day 0, the products were pressure treated (400 MPa for either 180 or 360 seconds) and microbial populations were determined in triplicate at ca. 1-2 hours after pressure treatment (ca. 2-3 hours after inoculation), using the same dilution and plating procedures as the non-HHP samples. All dilutions were made by adding 99 ml of Butterfield's phosphate buffer to each 11 gram sample, stomaching, making further dilutions as needed, and then plating on MOX agar via direct plating. The MOX plates for all treatments were incubated for 48 hours at 35°C then examined for the presence or absence of growth. All counts were run in triplicate for each replication, with each measurement converted to a logarithmic scale and averaged to give log CFU/gram for each replication. All initial dilutions (1:10) of test samples were stored in snap-cap cups at 4.4°C for later enrichment of the sample, if needed. An uninoculated, negative sample was also prepared for all treatments for each of the testing days.

If no colonies were detected on MOX agar (<10 CFU/gram), then enrichment of the pathogen was completed from the stored samples using USDA methods (USDA, 2009). Briefly, 25 ml of dilution sample was added to 225 ml of UVM broth and incubated for 24 hours at 30°C, then 0.1 ml of UVM broth/sample was transferred into 10 ml of Fraser broth with 0.1 ml ferric ammonium citrate and incubated for 48 hours at 35°C. Tubes were evaluated for the presence (positive) or absence (negative) of a darkening color. The positive enrichment was considered as 1 log CFU/g and the

negative enrichment was considered as  $-0.39 \log \text{CFU/g}$  (25 ml of total 110 ml sample was enriched {22%}, and 22% of original 11g slice is 2.42 g, therefore less than 1 CFU divided by 2.42 g equals  $<0.41 \text{CFU/gram}$  or  $<-0.39 \log \text{CFU/g}$ ). Enrichment samples were also streak plated onto MOX and incubated 48 hours at  $35^{\circ}\text{C}$  for confirmation. All enriched samples were recorded as positive (darkened color) or negative (no color change). A negative sample means there was  $<0.41 \text{CFU/gram}$  of the pathogen present in the sample. A positive sample means there was  $>0.41 \text{CFU/g}$ , but less than the direct plate (i.e.  $<10 \text{CFU/g}$ ). Therefore, for the numerical count, a positive sample was assumed to be a count of 10 CFU/g and negative sample was assumed to be 0.41 CFU/g.

Along with typical growth on MOX selective media, confirmatory tests were completed using Rapid'*L.mono*<sup>TM</sup> (Bio-Rad Laboratories, Marnes-la-Coquette, France).

**High-pressure equipment and conditions.** Samples that were processed under high hydrostatic pressure used a Quintus® Type QFP 35L-600 unit (Avure Technologies, Kent, WA). Pressure treatment was carried out at 400 MPa for 3 minutes or 6 minutes (not inclusive of come-up time of ca. 90 seconds, with an almost instantaneous depressurization) at a product temperature of  $5^{\circ}\text{C}$  and a starting vessel temperature of  $17^{\circ}\text{C} (\pm 2^{\circ}\text{C})$  with water as the surrounding pressure-transmitting medium. All samples for each replication were processed together after inoculation on day 0 and then samples at  $10^3$  inoculation were stored at  $4.4^{\circ}\text{C}$  for the remainder of the experiment.

**Chemical analyses.** Samples were analyzed for proximate composition of moisture (3), crude protein (5), and crude fat (4). Measurement of pH (93), residual nitrite (24), residual nitrate (25), and NaCl (26) were also conducted.

**Statistical analysis.** Results were analyzed using the proc glm statement of SAS (SAS 9.2, SAS Institute Inc., Cary, NC, 2008). For specific model effects ( $p < 0.05$ ), differences between least squares means were determined using the lsd procedure. For the 3 log inoculation analysis, log reduction was analyzed within type of formulation (natural or conventional) and HHP treatment. The fixed effects of formulation, days of shelf-life and replication and the interaction of formulation and days of shelf-life were used in the analysis. For the 5 log inoculation analysis, log reduction was analyzed within type of formulation (natural or conventional). The fixed effects of formulation, HHP time and replication and the interaction of formulation and HHP time were used in the analysis. For the analysis of pH, and the compositions of moisture, fat, protein and salt, formulation and replication served as fixed effects in the model. For the analysis of residual nitrite and nitrate, formulation, days of shelf-life and replication served as fixed effects. The interaction of formulation and days of shelf-life was also included in the model as an interaction. For the analysis of nitrite change due to HHP, the change in nitrite from before HHP to after HHP was calculated and analyzed using the fixed effects of formulation and replication.

## RESULTS AND DISCUSSION

**Conventional formulations.** The reduction in *L. monocytogenes* numbers for conventional formulas after inoculation with 5 log *L. monocytogenes* are shown in Table 3. For treatments without HHP, in comparison to the conventional control formulation, the addition of LM220 gave a significant reduction ( $p < 0.05$ ) of *L. monocytogenes* of 1.29 log CFU/g, LAE spray gave a reduction of 2.37 log CFU/g which was not significantly

different ( $p>0.05$ ) than LM220 treatment, and the combination of LM220 + LAE gave 3.44 log CFU/g which was significantly greater ( $p<0.05$ ) than LM220 but not significantly different ( $p>0.05$ ) from the LAE spray.

TABLE 3. *Log<sub>10</sub> reduction in L. monocytogenes in conventional formulations vs. no HHP control after inoculation with a 5-strain mixed culture of L. monocytogenes at a level of 10<sup>5</sup> CFU/g and no HHP (ingredient effect) or treatment with 400 MPa of high hydrostatic pressure for 3 or 6 minutes (ingredient plus HHP effect).*

Treatments	NO HHP CFU/g decrease	3 min. HHP CFU/g decrease	6 min. HHP CFU/g decrease
Control	0.00 <sup>ax</sup>	1.08 <sup>ay</sup>	1.33 <sup>ay</sup>
LM220 Ingredient	1.29 <sup>bx</sup>	2.75 <sup>by</sup>	2.64 <sup>by</sup>
LAE Spray	2.37 <sup>bcx</sup>	3.17 <sup>bcx</sup>	3.35 <sup>cx</sup>
LM220+LAE	3.44 <sup>cx</sup>	4.43 <sup>cx</sup>	4.43 <sup>dx</sup>
Standard Error	0.20	0.35	0.35

<sup>a,b,c,d</sup> Means with different superscripts show differences among LS means in columns ( $p<0.05$ )

<sup>x,y</sup> Means with different superscripts show differences among LS means in ROWS ( $p<0.05$ ).

For treatments with 3 minute HHP (Table 3), comparing the treatments to the control formulation without HHP, the control had 1.08 log CFU/g reduction in *L. monocytogenes*, which was significantly larger ( $p<0.05$ ) than the control treatment without HHP. The LM220 treatment had a reduction of 2.75 log CFU/g which was significantly greater ( $p<0.05$ ) than the control at 3 minutes HHP and also significantly greater ( $p<0.05$ ) than the LM220 treatment with no HHP. The LAE spray treatment had a reduction of 3.17 log CFU/g which was significantly larger ( $p<0.05$ ) than the control at 3 minute HHP, but not significantly different ( $p>0.05$ ) than the LM220 treatment at 3 minute HHP and not significantly different ( $p>0.05$ ) than the LAE spray treatment with no HHP. The LM220+LAE treatment had a reduction of 4.43 log CFU/g which was



significantly greater ( $p<0.05$ ) than the control and LM220 3 minute HHP treatments, but not significantly different ( $p>0.05$ ) than either the LAE spray with 3 minute HHP or the Danisco + LA treatment without HHP. All treatments with 6 minute HHP were not significantly different ( $p>0.05$ ) than the respective treatments with 3 minute HHP.

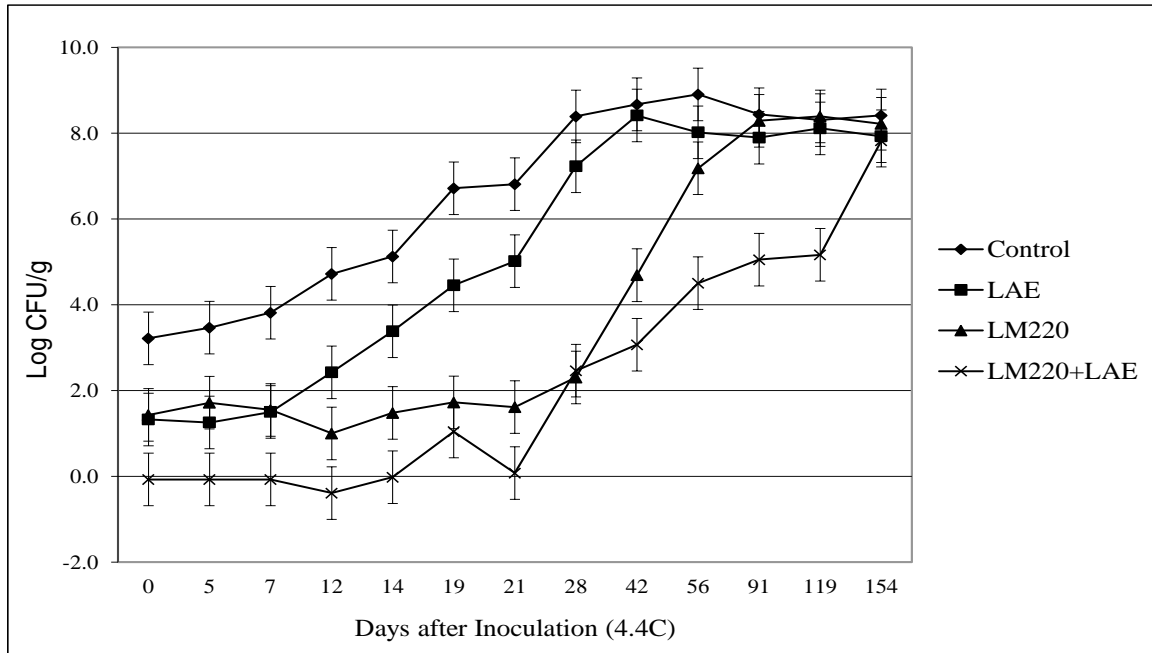


FIGURE 1. Least squares means by treatment for conventional formulations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g with no HHP treatment and evaluation through a 154-day storage period.

For the conventional formulations with 3 log inoculation without HHP, the numbers of *L. monocytogenes* over the 154 day storage period are shown in Figure 1. At day 0, the control treatment was significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers than all other treatments and this significant difference continued through day 21. From day 21 through the end of the study there was no significant difference ( $p>0.05$ ) between the conventional control and the LAE treatments. The LM220 treatment and the LAE treatment were not significantly different at day 0 through day 7

and were also not significantly different from day 56 through day 154. The LAE+LM220 treatment had significantly less *L. monocytogenes* than LM220 on days 5, 12, 56, 91, and 119. At day 154 all treatments were not significantly different ( $p>0.05$ ) and all had *L. monocytogenes* numbers of about 8 log CFU/g.

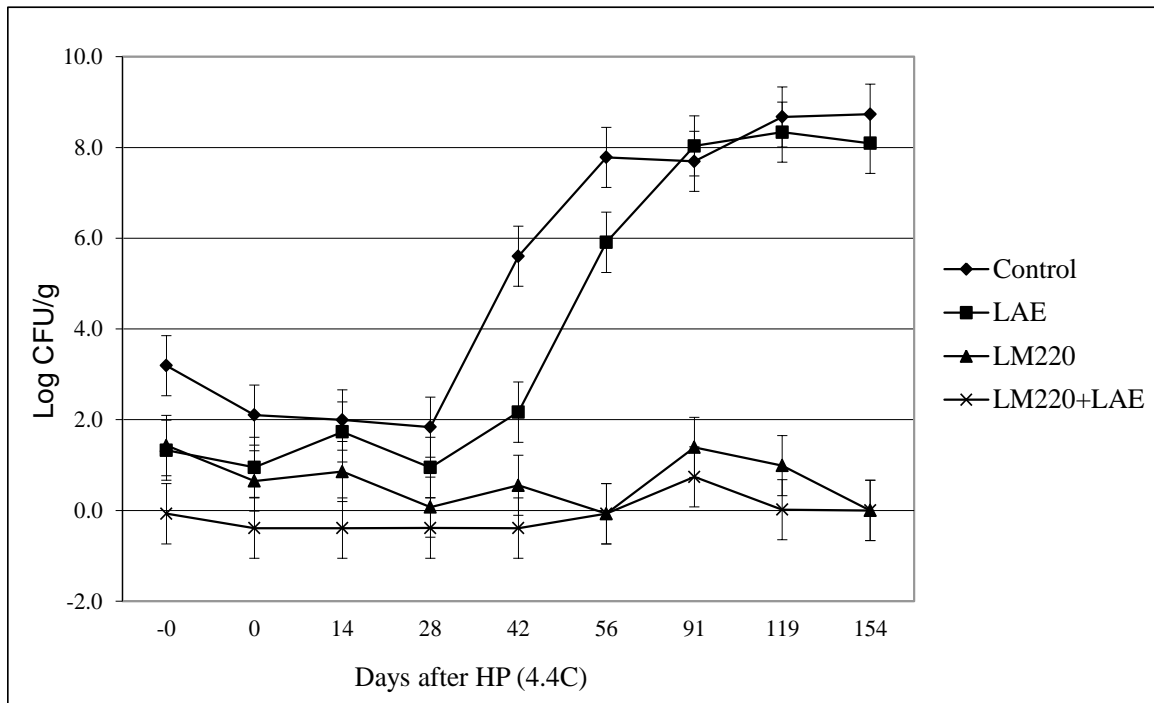


FIGURE 2. Least squares means by treatment for conventional formulations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g followed by HHP treatment at 400 MPa for 3 minutes and evaluation through a 154-day storage period.

The numbers of *L. monocytogenes* in conventional formulations with 3 log inoculation and either 3 minutes or 6 minutes at 400 MPa HHP are shown in Figures 2 and 3, respectively. On day 0, the control formulation after HHP had significantly greater ( $p<0.05$ ) numbers of *L. monocytogenes* than all other treatments after 6 minute HHP and for all treatments except LAE after 3 minute HHP. On day 0, for the 3 and 6 minute HHP times, the LAE, LM220, and LAE+LM220 treatments were not significantly

different ( $p<0.05$ ) in numbers of *L. monocytogenes*. Through the length of the study, the LAE+LM220 treatment had significantly less ( $p<0.05$ ) *L. monocytogenes* than the conventional control for both HHP times, except for day 28 for 6 minute HHP ( $p>0.05$ ). The LAE+LM220 treatment was not significantly different ( $p>0.05$ ) than the LM220 treatment for either 3 or 6 minute HHP at any of the days of evaluation. For the 3 minute HHP, the control was not significantly different ( $p<0.05$ ) in *L. monocytogenes* numbers from the LAE treatment on days 14, 28, 91, 119, and 154, but was significantly greater ( $p<0.05$ ) on days 42 and 56. The LAE treatment was not significantly different ( $p>0.05$ ) from the LM220 treatment on days 0 through 42, but was significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers through the remainder of the test.

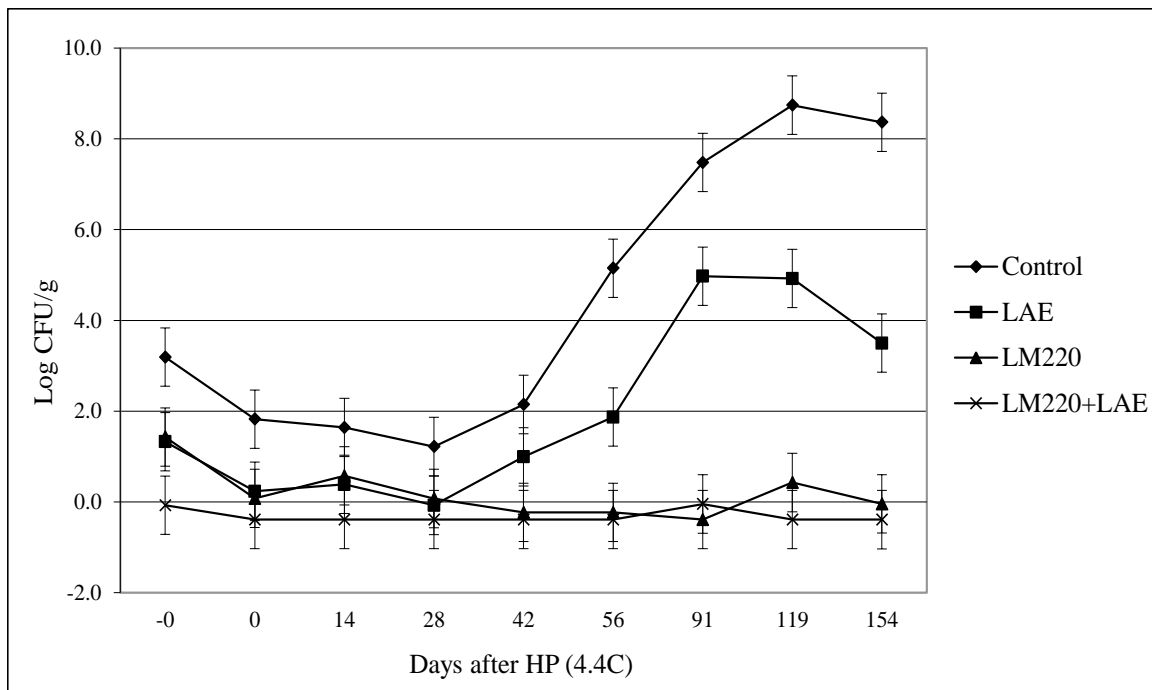


FIGURE 3. Least squares means by treatment for conventional formulations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at a level of  $10^3$  CFU/g (day 0) followed by HHP treatment at 400 MPa for 6 minutes and evaluation through a 154-day storage period.

For the 6 minute HHP treatment (Figure 3), the conventional control and LAE

treatments were not significantly different ( $p>0.05$ ) in *L. monocytogenes* numbers through day 42 and then from day 56 through 154 the control had significantly greater ( $p<0.05$ ) numbers of *L. monocytogenes* ( $p<0.05$ ) than all other treatments. Also at 6 minute HHP the LAE treatment was not significantly different ( $p>0.05$ ) than the LM220 or the LAE+LM220 treatments at days 0 through 42, but then was significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers from days 56 through 154.

**Natural formulations.** The reduction in *L. monocytogenes* numbers for natural formulas after inoculation with 5 log *L. monocytogenes* are shown in Table 4. For treatments without HHP, the addition of MOstatin did not give any significant change in *L. monocytogenes* numbers ( $p>0.05$ ) compared to the natural control without HHP. The MOstatin+LAE and MOstatin+OA treatments had 0.81 and 0.67 log CFU/g reduction in *L. monocytogenes* numbers compared to the control, which was a significantly greater reduction ( $p<0.05$ ) than the control and the MOstatin only treatments, but not significantly different ( $p>0.05$ ) from one another. For the treatments with 3 minute HHP, comparing the treatments to the natural control formulation without HHP, the control had 0.88 log CFU/g reduction in *L. monocytogenes*, which was significantly greater ( $p<0.05$ ) than the control treatment without HHP. The MOstatin treatment had a reduction of 1.10 log CFU/g which was not significantly different ( $p>0.05$ ) than the control at 3 minutes HHP but was significantly greater ( $p<0.05$ ) than the MOstatin treatment with no HHP. The MOstatin+LAE treatment had a reduction of 2.16 log CFU/g which was significantly greater ( $p<0.05$ ) than the control and MOstatin treatments at 3 minute HHP and significantly greater ( $p<0.05$ ) than the MOstatin+LAE treatment with no HHP. The MOstatin+OA treatment had a reduction of 2.38 log CFU/g which not

significantly different ( $p>0.05$ ) than the MOstatin+LAE treatment but was significantly greater ( $p<0.05$ ) than 3 minute HHP for natural control and MOstatin treatments. The 3 minute HHP MOstatin+LAE treatment also had a significantly greater ( $p<0.05$ ) reduction in *L. monocytogenes* numbers than the same treatment without HHP. The natural product treatments with 6 minute HHP were not significantly different ( $p>0.05$ ) in *L. monocytogenes* numbers than their matching treatments with 3 minute HHP.

TABLE 4. *Log<sub>10</sub> reduction in L. monocytogenes in natural formulations vs. no HHP natural control after inoculation with a 5-strain mixed culture of L. monocytogenes at a level of 10<sup>5</sup> CFU/g and no HHP (ingredient effect) or treatment with 400 MPa of high hydrostatic pressure for 3 or 6 minutes (ingredient plus HHP effect).*

Treatments	NO HHP CFU/g decrease	3 min. HHP CFU/g decrease	6 min. HHP CFU/g decrease
Natural Control	0.00 <sup>ax</sup>	0.88 <sup>ay</sup>	1.16 <sup>ay</sup>
MOstatin Ingredient	-0.11 <sup>ax</sup>	1.10 <sup>ay</sup>	1.43 <sup>ay</sup>
MOstatin+LAE Spray	0.81 <sup>bx</sup>	2.16 <sup>by</sup>	2.13 <sup>by</sup>
MOstatin+OA Spray	0.67 <sup>bx</sup>	2.38 <sup>by</sup>	2.17 <sup>by</sup>
Standard Error	0.08	0.16	0.16

<sup>a,b,c,d</sup> Means with different superscripts show differences among LS means in columns ( $p<0.05$ )

<sup>x,y</sup> Means with different superscripts show differences among LS means in ROWS ( $p<0.05$ ).

For the natural formulations with 3 log inoculation without HHP, the numbers of *L. monocytogenes* over the 154 day storage period are shown in Figure 4. At day 0, the natural control treatment and MOstatin treatment were not significantly different ( $p>0.05$ ) and were significantly greater in *L. monocytogenes* numbers ( $p<0.05$ ) than the MOstatin+OA and MOstatin+LAE treatments. The control treatment began to increase in *L. monocytogenes* numbers after day 0 and by day 7 *L. monocytogenes* numbers were significantly greater ( $p<0.05$ ) than the rest of the treatments and remained significantly

greater through the end of the 154 day study. The MOstatin treatment was significantly greater ( $p<0.05$ ) in *L. monocytogenes* numbers than the MOstatin+LAE treatment on days 0 through day 42 and on days 91 and 119. The MOstatin+OA was not significantly different ( $p>0.05$ ) from the MOstatin treatment at days 5 and 7, but then had significantly less ( $p<0.05$ ) *L. monocytogenes* than MOstatin treatment at days 12 through 28 and 56 through 119. The MOstatin+LAE and MOstatin+OA with no HHP were not significantly different ( $p<0.05$ ) in *L. monocytogenes* numbers throughout the entire 154 day study. The MOstatin+LAE and MOstatin treatments had a significant reduction ( $p<0.05$ ) in *L. monocytogenes* numbers from day 0 to day 154.

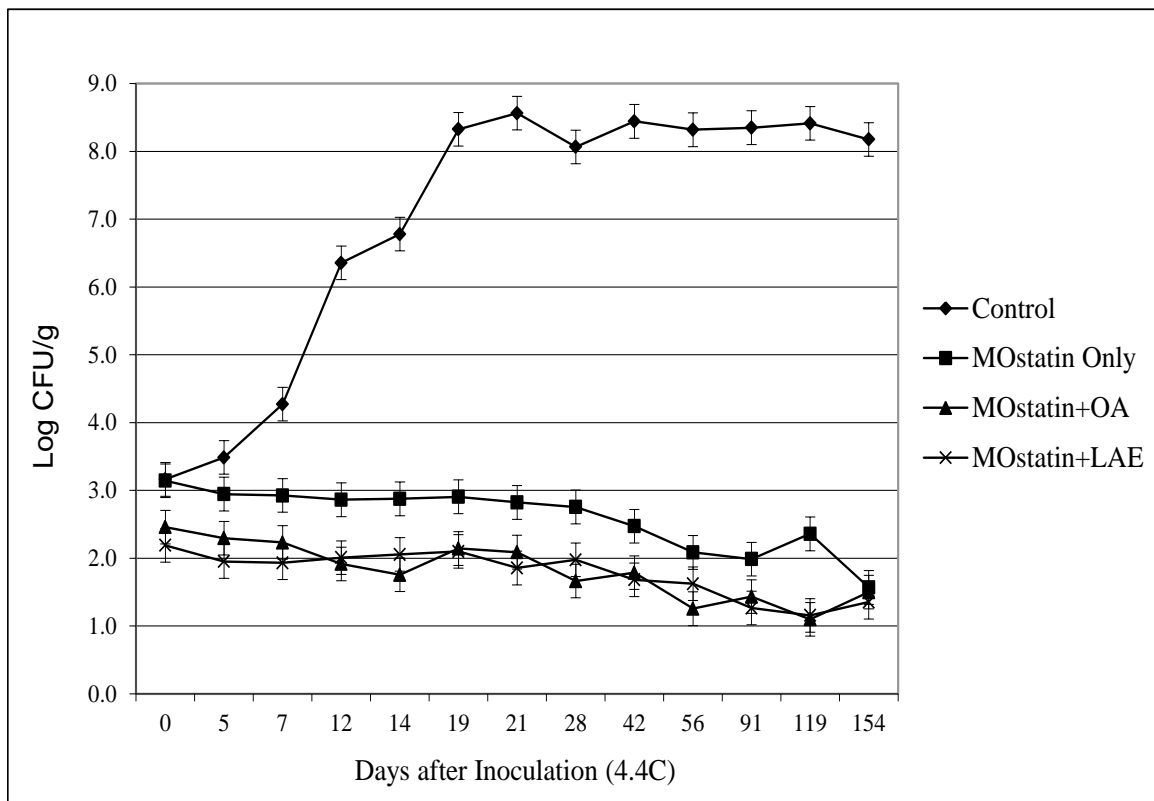


FIGURE 4. Least squares means by treatment for natural formulations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at  $10^3$  CFU/g with no HHP treatment.

The numbers of *L. monocytogenes* in natural formulations with 3 log inoculation

and either 3 minutes or 6 minutes of 400 MPa HHP are shown in Figures 5 and 6, respectively. At 3 minutes of HHP the control formulation was not significantly different ( $p < 0.05$ ) than the MOstatin treatment immediately after HHP (day 0), but then had significantly greater numbers of *L. monocytogenes* ( $p < 0.05$ ) through the rest of the study.

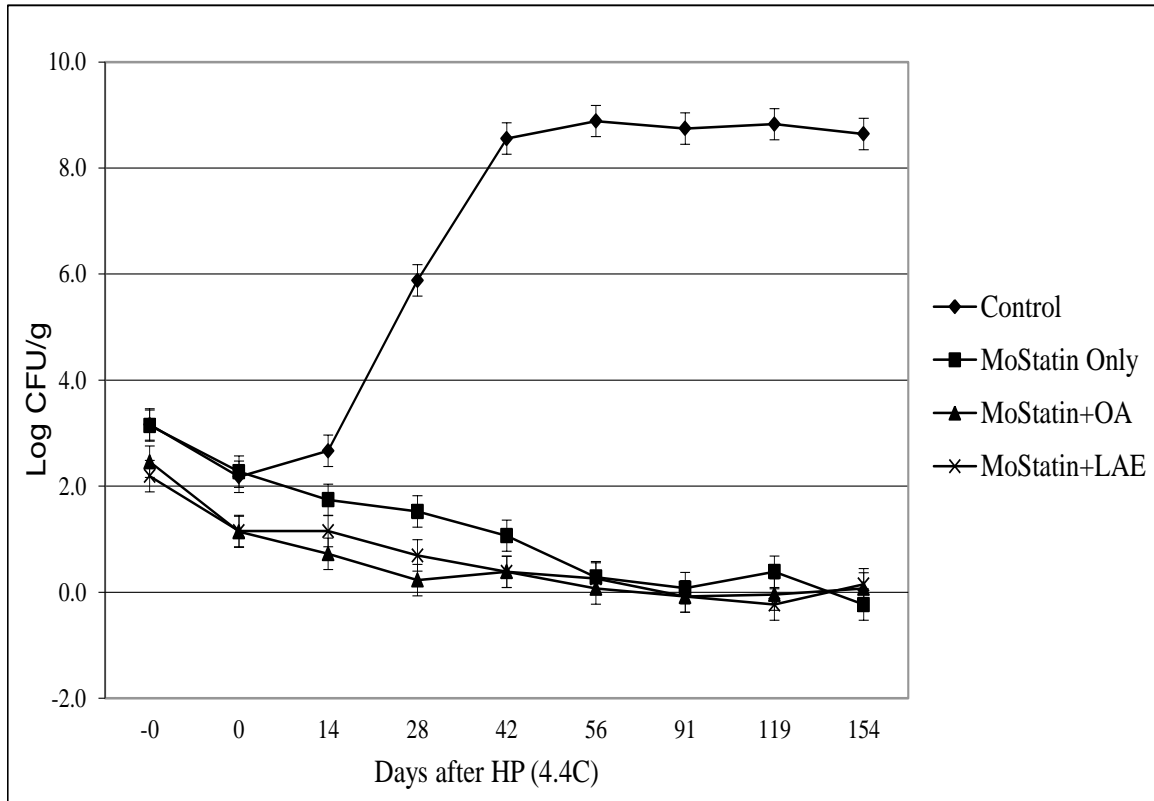


FIGURE 5. Least squares means by treatment for natural formulations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at  $10^3$  CFU/g followed by HHP treatment at 400 MPa for 3 minutes and evaluation through a 154-day storage period.

At 6 minutes of HHP there were no treatment differences ( $p > 0.05$ ) on days 0 and 14, but from day 28 through day 154, the natural control had a significantly greater number of *L. monocytogenes* ( $p < 0.05$ ) than the other treatments. The MOstatin+LAE, MOstatin+OA, and MOstatin treatments were not significantly different ( $p > 0.05$ ) at both HHP times on days 14 through the remainder of the 154 day study.

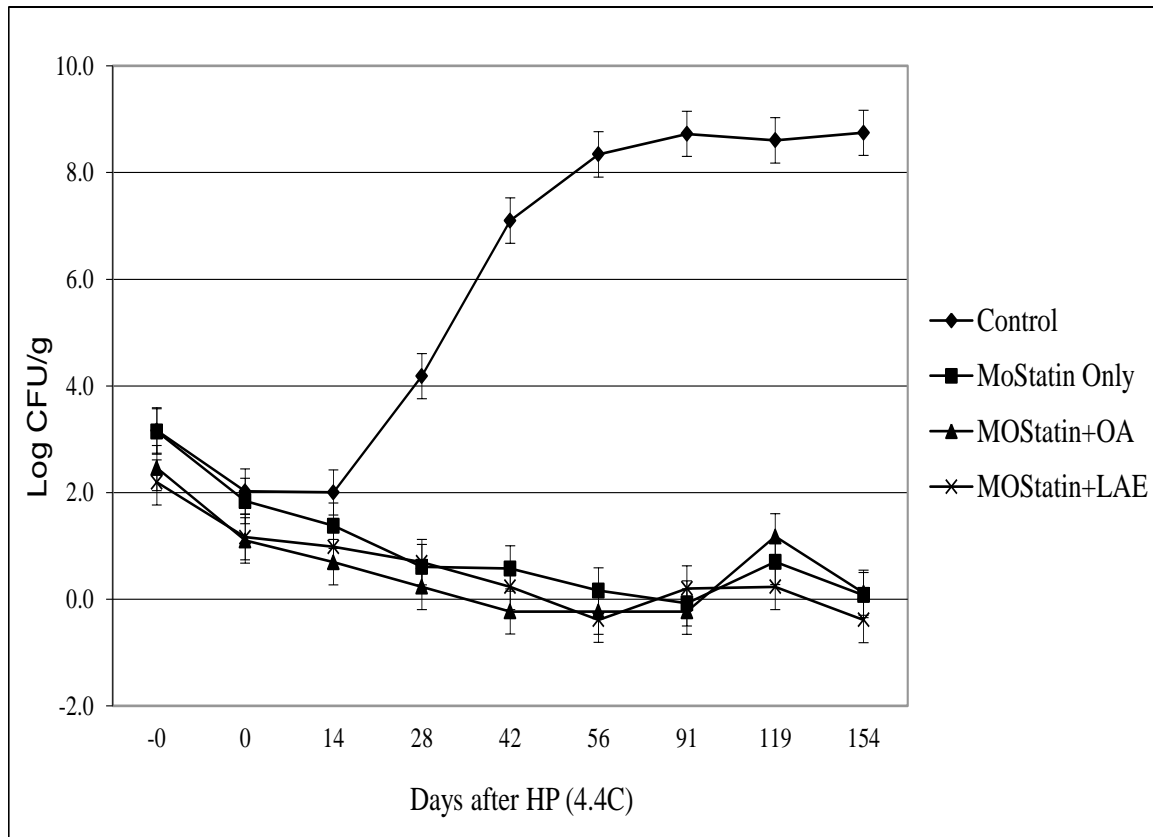


FIGURE 6. Least squares means by treatment for natural formulations after inoculation with a 5-strain mixed culture of *L. monocytogenes* at  $10^3$  CFU/g followed by HHP treatment at 400 MPa for 6 minutes and evaluation through a 154-day storage period.

**HHP Time Effect.** The least squares means for *L. monocytogenes* log CFU/g at a given HHP time throughout the study period is shown in Figure 7 (combination of both natural and conventional formulations). The least squares means for 0 minute HHP was significantly greater ( $p < 0.05$ ) in *L. monocytogenes* population compared to both 3 minutes or 6 minutes at all days except day 0 for 3 minutes, which trended towards significance ( $p < 0.10$ ). The average least squares mean for *L. monocytogenes* population was numerically greater for the 3 minute HHP time than the 6 minute HHP time at all days and trended greater ( $p < 0.10$ ) at days 56 and 154, but was not significantly different ( $p > 0.05$ ) on any of the days of the study.



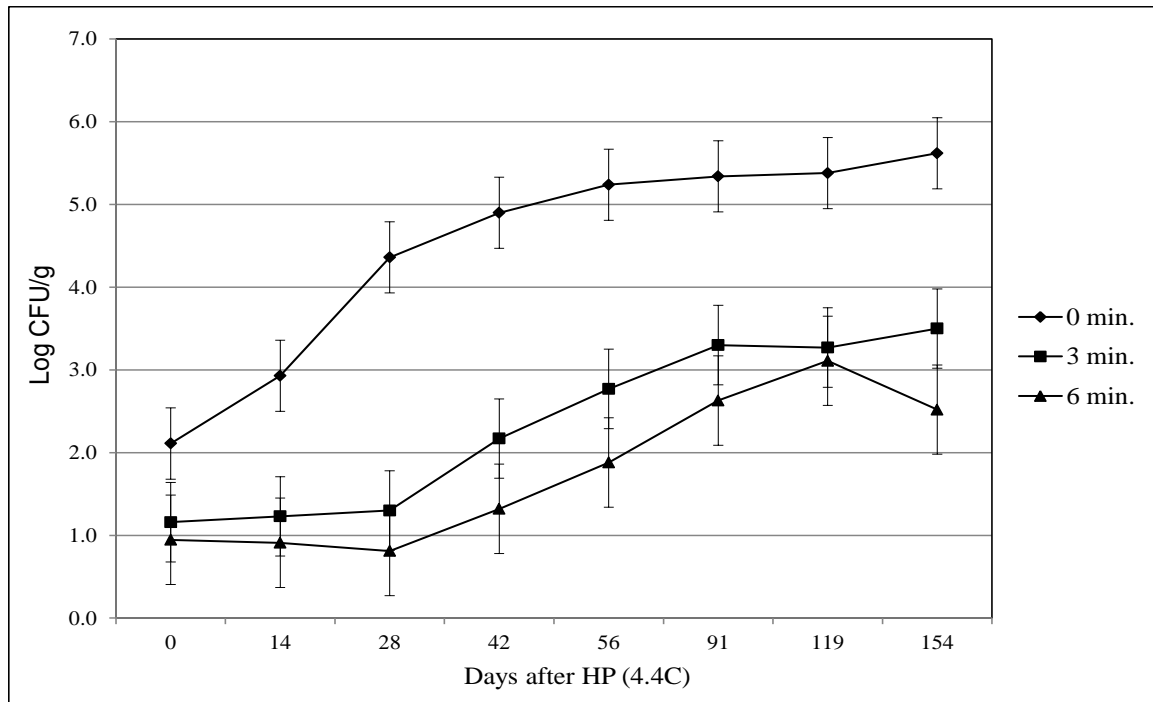


FIGURE 7. *Least squares means by HHP time after inoculation with a 5-strain mixed culture of L. monocytogenes at a level of  $10^3$  CFU/g followed by HHP treatment at 400 MPa for 0, 3, or 6 minutes, and evaluation through a 154-day storage period.*

**Product analyses.** The least squares means for residual nitrite before and after HHP for the four base formulations are shown in Table 5. There was no significant difference ( $p>0.05$ ) in the residual nitrite concentration before and after HHP for all treatments except the LM220 formula, which had significantly less residual nitrite after HHP. There were significant differences in residual nitrite concentrations between treatments as the conventional control was significantly greater ( $p<0.05$ ) in residual nitrite than all other formulations before and after HHP. The LM220 formulation and natural control formulations were not significant different ( $p>0.05$ ) in residual nitrite concentration and were both significantly greater ( $p<0.05$ ) than the MOstatin formula before HHP. The natural control and the MOstatin formulas were not significantly different ( $p<0.05$ ) in residual nitrite concentrations after HHP.

TABLE 5. *Least squares means for residual nitrite values (ppm) for treatments before and after HHP treatment at 600 MPa for 10 minutes as well as pH values.*

Treatments	Before HHP	After HHP
Conventional Control	79.3 <sup>cx</sup> ± 6.00	79.4 <sup>cx</sup> ± 8.2
LM220 Ingredient	49.4 <sup>bx</sup> ± 7.8	47.7 <sup>by</sup> ± 7.7
Natural Control	46.1 <sup>bx</sup> ± 2.2	45.9 <sup>abx</sup> ± 2.5
MOstatin Ingredient	38.9 <sup>ax</sup> ± 4.4	38.3 <sup>ax</sup> ± 3.8
Avg. LS Mean	53.4	52.8

<sup>a,b</sup> Means with different superscripts show differences among LS means in columns ( $p < 0.05$ ).

<sup>x,y</sup> Means with different superscripts show differences among LS means in ROWS ( $p < 0.05$ ).

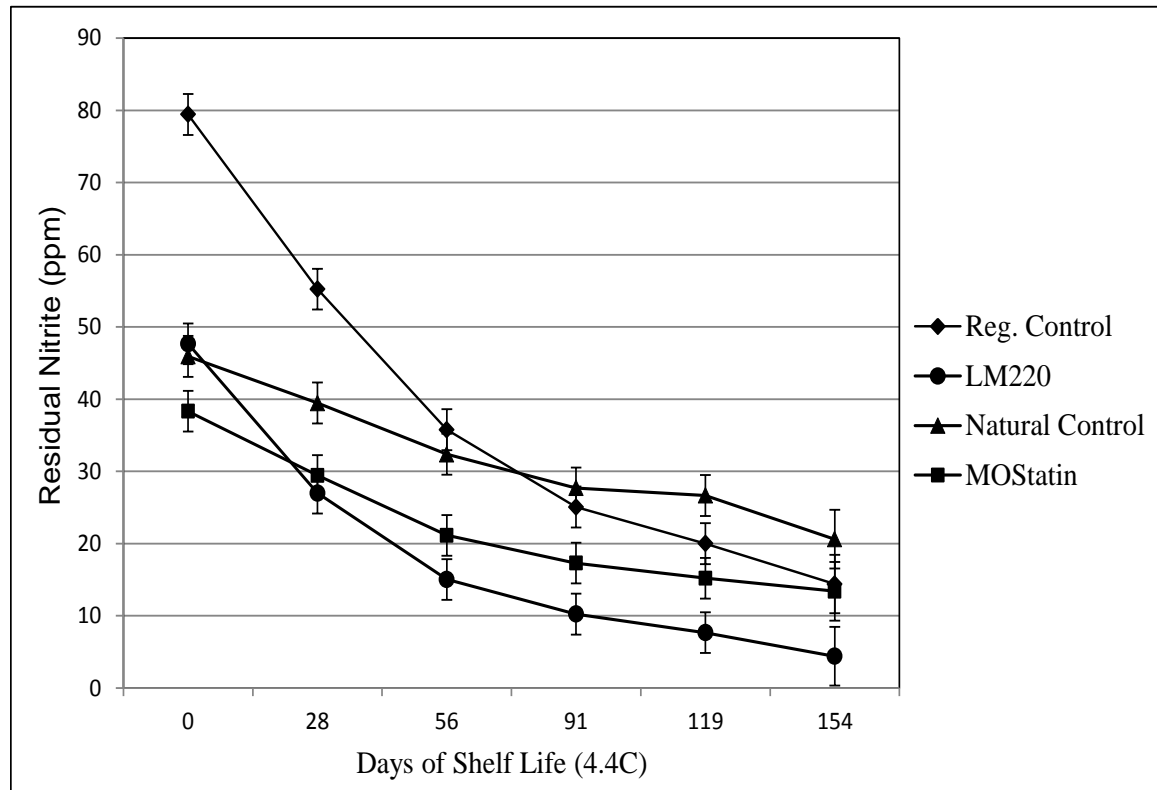


FIGURE 8. *Average residual nitrite level of formulations over shelf life (uninoculated samples with 10 minute HHP treatment).*

The residual nitrite concentrations over the storage period of the study for the four base formulations are shown in Figure 8. The conventional control formulation was

significantly greater ( $p < 0.05$ ) in residual nitrite than the remaining formulations for days 0 and 28. The conventional control was then not significantly different ( $p > 0.05$ ) than the natural control for days 56 and 91 and was significantly less ( $p < 0.05$ ) than the natural control and not significantly different ( $p > 0.05$ ) than the MOstatin treatment at days 119 and 154. The residual nitrite concentration of the LM220 formulation was not significantly different ( $p > 0.05$ ) than MOstatin at days 28 and 56 and then was significantly less ( $p < 0.05$ ) than all other formulations through the remainder of the study. For residual nitrate (Table 6) there was a significant day effect ( $p = 0.008$ ), but no significant effect of treatment ( $p > 0.05$ ), or interaction of treatment by day ( $p > 0.05$ ).

TABLE 6. *Least squares means of residual nitrate levels over shelf life of sliced, vacuum-packaged ham.*

	Day 0	Day 28	Day 56	Day 91	Day 119
Conventional Control	7.3±7.6	9.0±7.8	14.1±1.7	8.1±7.1	5.8±3.1
LM220 Ingredient	6.9±5.3	9.5±7.2	14.5±2.0	8.2±5.7	9.6±5.7
Natural Control	4.1±3.1	11.4±7.2	16.9±3.1	12.2±5.4	11.1±3.2
MOstatin Ingredient	6.6±3.7	11.7±9.2	15.4±5.6	12.1±3.1	11.1±2.8

The least squares means for proximate analyses for the four base formulations are shown in Table 7. There were no significant differences ( $p > 0.05$ ) in fat level between all treatments and no significant differences ( $p > 0.05$ ) in protein analyses between the two conventional formulations (Control and LM220) and between the two natural formulations (Natural Control and MOstatin). The pH of both of the control formulations was significantly greater ( $p < 0.05$ ) than the pH of the corresponding formulation with added ingredients. The salt concentration of the conventional control was significantly

less ( $p < 0.05$ ) than the LM220 formulation. The salt concentration of the natural control and the MOstatin formulation were not significantly different ( $p > 0.05$ ).

TABLE 7. *Least squares means for percent finished product moisture, fat, protein, and salt, as well as finished product pH.*

	Moisture	Fat	Protein	Salt	pH
Conventional Control	76.30 <sup>c</sup>	1.76 <sup>a</sup>	17.73 <sup>a</sup>	2.42 <sup>a</sup>	6.37 <sup>b</sup>
LM220	75.75 <sup>b</sup>	1.50 <sup>a</sup>	17.74 <sup>a</sup>	2.53 <sup>c</sup>	6.18 <sup>a</sup>
Natural Control	75.95 <sup>b</sup>	1.56 <sup>a</sup>	18.17 <sup>b</sup>	2.47 <sup>ab</sup>	6.42 <sup>c</sup>
MOstatin	75.48 <sup>a</sup>	1.63 <sup>a</sup>	18.08 <sup>b</sup>	2.51 <sup>bc</sup>	6.20 <sup>a</sup>
Std. Error	0.08	0.11	0.08	0.02	0.01

<sup>a,b,c</sup> Means with different superscripts show differences among LS means in columns ( $p < 0.05$ )

This study suggested that 400 MPa HHP without additional ingredients was inadequate to restrict growth of *L. monocytogenes*, as *L. monocytogenes* numbers in the control treatment for of both natural and conventional sliced RTE ham formulations grew to at or above the inoculum level within 42 to 56 days. The addition of LM220 ingredient to a conventional ham formulation gave a significant reduction in the number of *L. monocytogenes* in sliced RTE ham compared to a conventional control and this effect was enhanced by addition of LAE spray to the RTE product and by post-packaging HHP.

The additive effects of ingredients, spray treatment, and HHP were probably due to the ability of the ingredient and spray to cause initial damage to the *L. monocytogenes* cellular membrane before HHP was applied and the process of HHP then caused further membrane disruption thus decreasing the overall numbers of *L. monocytogenes*. LAE has been shown by other researchers to cause membrane damage in pathogenic

microorganisms (79). However, as also observed by others (53, 89) the treatment with LAE alone showed recovery of *L. monocytogenes* and the ability grow to large numbers within a short period of time. This effect was observed even with the use of 400 MPa pressure in conventional formulations. The conventional formulations subjected to HHP with added LM220 and with or without LAE were able to further inhibit *L. monocytogenes* over the storage period and kept numbers of organisms below the initial inoculum level.

For natural formulations, the use of MOstatin alone or in combination with OA or LAE maintained *L. monocytogenes* numbers below the inoculum level throughout the study period even without use of HHP. With HHP, the addition of MOstatin gave no additional initial reduction in counts above what HHP alone gave. However when either OA or LAE were added in combination with MOstatin, there was an immediate effect of reduced numbers of *L. monocytogenes* and a lasting inhibition over the 154-day storage period.

The effect of pH may have also played a role in reducing *L. monocytogenes* counts, as shown in a previous study (64) and by other researchers (10, 96). The addition of MOstatin in the natural formulations and LM220 in the conventional formulation both decreased the pH of the finished RTE product and may have contributed to the decreased numbers of *L. monocytogenes* in these treatments.

Another significant point is that products that supported the growth of *L. monocytogenes* in this study maintained consistent numbers of this pathogen for a long period. In the natural and conventional formulations without HHP, *L. monocytogenes* numbers remained >8 log CFU/g for over 125 days. In evaluation of product containing

*L. monocytogenes* from foods consumed by patients with listeriosis, researchers have speculated that *L. monocytogenes* numbers found at low levels have probably decreased from greater initial levels and therefore low numbers of *L. monocytogenes* do not cause illness (22, 42, 66). This study suggests that numbers of *L. monocytogenes* remain in a stationary phase for a long period and may not be significantly decreased as quickly as some have speculated. This ability of *L. monocytogenes* to maintain a long stationary phase at a given level of contamination could support the claim of other researchers that some strains of *L. monocytogenes* could potentially cause severe illnesses even at low levels in foods (21, 33, 61, 67, 70, 72).

This study shows that it is possible to use high hydrostatic pressure as low as 400 MPa in combination with other ingredients can achieve a significant initial reduction in numbers of *L. monocytogenes* and maintain continued suppression throughout shelf life. Because antimicrobial ingredients and sprays were used at or near FSIS limits, further work is needed to assess the sensory acceptability of various product formulations and changes in efficacy if levels of ingredients or sprays need to be reduced to meet sensory targets.

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## CHAPTER 5 - OVERALL CONCLUSIONS

*Listeria monocytogenes* is a foodborne pathogen of great concern for RTE meat items due to its ubiquitous nature, potential to grow at low temperatures, and its high fatality rate compared with other pathogens. The USDA has recognized *L. monocytogenes* as an adulterant and has a zero-tolerance for the pathogen in ready-to-eat meat (RTE) items. Meat processors have implemented many control measures to minimize risk of *L. monocytogenes* in RTE products over the past several years and the number of reported positive samples of meat items with *L. monocytogenes* has shown a steady decline over the last several years. Despite this effort there is still a risk for *L. monocytogenes* contamination in RTE items that are handled after cooking and recalls of products contaminated with *L. monocytogenes* still occur several times per year.

The research studies described in this document were in the broad area of the processing intervention high hydrostatic pressure (HHP). HHP has been adopted by some food processors to minimize pathogen risks and to decrease growth of spoilage organisms. The first research study presented here showed that HHP is a technology capable of decreasing the number of *L. monocytogenes* organisms by 3-4 log CFU/g in conventionally-cured processed meats. There was a great deal of prior literature suggesting that turkey was an excellent growth media for *L. monocytogenes* and that it grew more readily in turkey meat than in other species. This hypothesis was not supported by this study when all other formulation and processing variables were held constant as the study here showed that turkey and ham (pork) had very similar levels of growth without HHP. The inclusion of nitrite was the key variable in the study that caused a decrease in growth of *L. monocytogenes*. However, when formulations were



processed via 600 MPa HHP after inoculation with  $10^3$  log CFU/g of *L. monocytogenes*, the level of *L. monocytogenes* was decreased to below the detection limit in all treatments and remained below the detection limit in all treatments for at least 119 days after treatment.

A second study showed that use of a reduced level of pressure at 400 MPa was not adequate in decreasing the number of *L. monocytogenes* by  $>1$  log CFU/g. The addition of nitrite from either a natural source (pre-converted vegetable juice powder), at 50 or 100 ppm, or from sodium nitrite, at 100 or 200 ppm, were equal in their inhibition of *L. monocytogenes*. For some unexplained reason the greater concentrations of natural nitrite (150 and 200 ppm) that used HHP had, towards the end of storage, greater growth of *L. monocytogenes* than all other treatments. Some hypotheses were presented, including a greater pH level in the treatments with a greater concentration of natural nitrite, but further research is needed to understand the root cause of the greater level of growth in these treatments.

The third study evaluated the use of the reduced pressure level of 400 MPa HHP in combination with antimicrobial compounds to provide added reduction of *L. monocytogenes* above what each of the interventions could achieve on their own in cured meats, using either natural or conventional formulations. The addition of antimicrobial ingredients to the formulations or use of post-lethality antimicrobial sprays in conventional items gave a 1-2 log CFU/g reduction of *L. monocytogenes* numbers, while combining each of these with 400 MPa HHP gave about a 3 log CFU/g reduction in *L. monocytogenes* numbers. However the combination of ingredient, spray, and 400 MPa HHP gave a  $>4$  log CFU/g reduction in *L. monocytogenes* in conventional products.

Similar effects were witnessed in natural products but at reduced levels of efficacy, as the combination of ingredient, spray, and 400 MPa HHP gave an initial 2.1-2.4 log CFU/g reduction in *L. monocytogenes*. In conventional and natural products, when an antimicrobial ingredient was added (Danisco LM220 for conventional or MOStatin for natural) in combination with HHP, the level of *L. monocytogenes* continued to fall over the extended shelf life (182 days) of the sliced ham.

Overall this research shows the benefits of high hydrostatic pressure in controlling *L. monocytogenes* by serving as a post-packaging treatment capable of decreasing the number of *L. monocytogenes* by over 3 log CFU/g at 600 MPa. The research also showed that the meat species had no effect on growth of *L. monocytogenes* with or without the use of HHP. The use of nitrite in a formula inhibited growth of *L. monocytogenes*, but was not listericidal and the use of natural nitrite at the current usage levels of 50-100 ppm showed similar *L. monocytogenes* growth compared to conventional sodium nitrite cured products. Also, the potential to use pressure levels below the current standard of 600 MPa for RTE products is possible in conventionally cured items using a combination of antimicrobial ingredients in the formulation and post-lethality antimicrobial sprays to achieve an *L. monocytogenes* inactivation level that is equal or greater than 600 MPa without use of antimicrobials. However in the natural products, the tests of currently available natural antimicrobials and natural post-lethality sprays along with 400 MPa HHP suggests that *L. monocytogenes* reduction in numbers is less than can be achieved by 600 MPa without addition of antimicrobial ingredients.

### **Future Research**

Processed meats include a wide variety of products with different flavor nuances and expectations. Further research in the area of sensory testing and process optimization is needed to understand the level of antimicrobials that can be added to various formulations and still maintain the expected sensory characteristics of the product. Also further work is needed to explore other antimicrobial compounds that could potentially disrupt cell membranes of Gram-positive organisms and therefore could provide additive or synergistic effects with high hydrostatic pressure. Currently the flavor impact of vegetable juice powder limits the level of use to below 100 ppm of added nitrite in the finished product. Further research is needed to understand the growth rate of *L. monocytogenes* in products using greater concentrations of natural nitrite to assure that there are no characteristics that cause unexpected microbial growth in these items, as suppliers will continue to explore ways to minimize the flavor impact from this ingredient.

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